PHARMACOKINETICS AND METABOLISM OF MZPES, A NOVEL LIPOPHILIC ANTIFOLATE

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Thesis Summary

m-Azidopyrimethamine ethanesulphonate salt (MZPES) is a new potent dihydrofolate reductase inhibitor designed to be both lipophilic and rapidly biodegradable. The drug is active against some methotrexate-refractory cell lines and against a broad spectrum of malignant cells in murine models. The pharmacokinetics of the drug were evaluated in the mouse, rat and man. A specific analytical method was developed to allow determination of MZP (the free base of MZPES) and its putative metabolite m-amino-pyrimethamine (MAP) in plasma, urine, faeces and tissues. Analytical methodology involved solvent extraction followed by reversed-phase ion-pair high pressure liquid chromatography. Mice were dosed at 10 and 20 mg/kg IP and 10 mg/kg PO. Absorption was rapid from both sites with a mean plasma elimination half-life of 4 hours. Oral bioavailability, relative to intraperitoneal injection, exceeded 95% in the mouse. MZP attained concentrations in mouse tissues 4 to 14 fold greater than those found in plasma and penetrated the blood-brain barrier effectively Following intraperitoneal administration of MZP to the rat the recovery of MZP and MAP in urine and faeces was 14% during 72 hours. MZPES was formulated for a phase I clinical evaluation as a 1% w/v aqueous solution and was administered by IV infusion in 5% dextrose over 1 hour. The drug obeyed 2-compartment kinetics with a central compartment volume of 27 litres and a volume of distribution of 118 litres. Plasma distribution and elimination half-lives were 0.27 and 34 hours respectively and plasma clearance was 7.5 L/hr. MZP was removed from plasma more rapidly than the prototypic lipophilic dihydrofolate reductase inhibitor metoprine (half-life 216 hours). The pharmacokinetics of MZPES showed no dose-dependency over the dose-range studied (27 to 460 mg/m^2). The dose-limiting toxicity was nausea and vomiting. The short half-life of the drug should allow easy assessment of the optimum dose and schedule of administration.

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ABBREVIATIONS AND SYMBOLS

A(1%, 1 cm)	Specific absorbance
AIDS	Aquired immunodeficiency syndrome
AUC	Area under plasma concentration-time curve
cm	Centimetre
CNS	Central nervous system
conc	Concentration
Cp_{max}	Maximum plasma concentration
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dTMP	Thymidylate
dUMP	Deoxyuridylate
F	Oral bioavailability
g	Acceleration of free fall
GC	Gas chromatography
HMT	Histamine N-methyltransferase
HPLC	High pressure liquid chromatography
hr	Hour
ID_{50}	Drug concentration which inhibits enzyme activity or rate of cell proliferation by 50%
ILS	Increase in lifespan in comparison with control
IP	Intraperitoneal
IV	Intravenous
k^1	Column capacity factor
kg	Kilogram
K_i	Drug-enzyme binding constant
L	Litre
LD_{10}	Dose which kills 10% of a population
LD_{50}	Dose which kills 50% of a population

Log P	Log octanol-water partition coefficient
M	Molar
MAP	m-Aminopyrimethamine
MAP-Ac	m-Acetamidopyrimethamine
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MS	Mass spectrometry
MTX	Methotrexate
MZPES	m-Azidopyrimethamine ethanesulphonate salt
n	Number of observations
NADPH	Reduced nicotinamide-adenine dinucleotide phosphate
NCI	National Cancer Institute
ng	Nanogram
nm	Nanometre
NMR	Nuclear magnetic resonance
PO	By mouth
r	Correlation coefficient
R_f	Rate of movement of solute/ rate of movement of mobile phase
rt	Retention time
SC	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulphate
$t_{\frac{1}{2}}$	Half-life
THF	Tetrahydrofolate
TLC	Thin layer chromatography
T_{max}	Time at which maximum concentration was reduced
UV	Ultraviolet
V_d	Volume of distribution
W-256	Walker 256

Look to your health; and if you have it, praise God, and value it next to a good conscience; for health is the second blessing that we mortals are capable of; a blessing that money cannot buy.

Izaak Walton Compleat Angler, pt.i, ch.21

1. INTRODUCTION

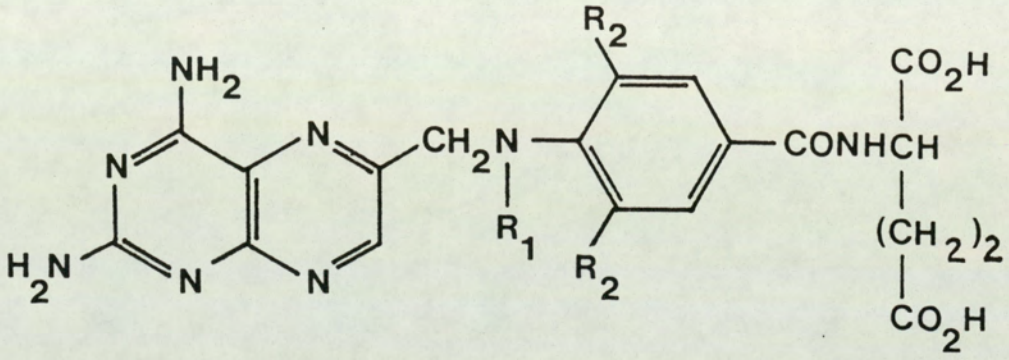
1.1 General

A significant advance in the systemic treatment of cancer was the discovery in 1948 by Farber et al (1) that the antifolate aminopterin (Figure 1.1) regularly produced remissions in acute leukaemia in children. Early successes such as this led to the development of a profusion of chemotherapeutic agents and today over 30 such drugs are in use in the clinic. Chemotherapy can produce beneficial palliation, prolongation of life and even cures in some forms of malignant disease, and it is a useful adjunct to surgery or radiotherapy (2).

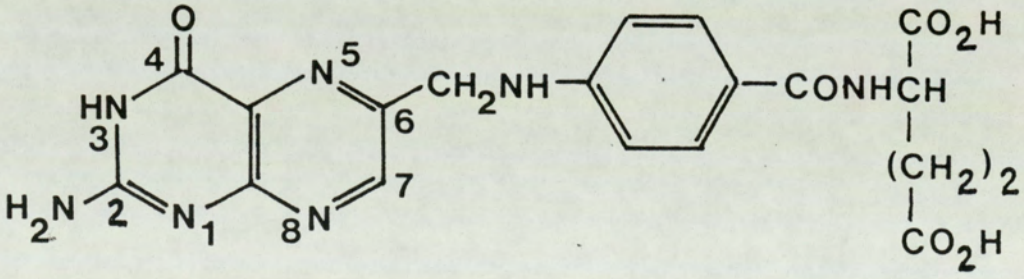
Farber's work was followed closely by the synthesis of another water-soluble antifolate (3), methotrexate (MTX, Figure 1.1), which is a major anticancer agent today. The development of cellular resistance to MTX, poor activity against many solid tumours and minimal penetration into the CNS (2,4,5,6) stimulated investigation of small "non-classical" lipophilic antifolates. These drugs have potentially favourable cellular transport properties because they can easily penetrate cell membranes, possibly by passive diffusion (7-9), in contrast to MTX, which enters cells by an energy-requiring carrier-mediated active transport process (10). The non-classical antifolates are active against many MTX-resistant cell lines and solid tumours (11-15) and they enter the CNS (16-19) but their adverse effects are considerable. They suppress bone marrow and exhibit gastrointestinal, skin and CNS toxicities (20-27). The mucosal, dermal and neurological sequalae may result from inhibition of histamine metabolism (28,29). The haematological and gastrointestinal effects are folate-related and are common to MTX and the lipophilic antifolates (4,20-24, 26) but the bone marrow and gut may

Figure 1.1 Chemical structures of aminopterin, methotrexate, 3',5'-dichloromethotrexate, folic acid and folinic acid

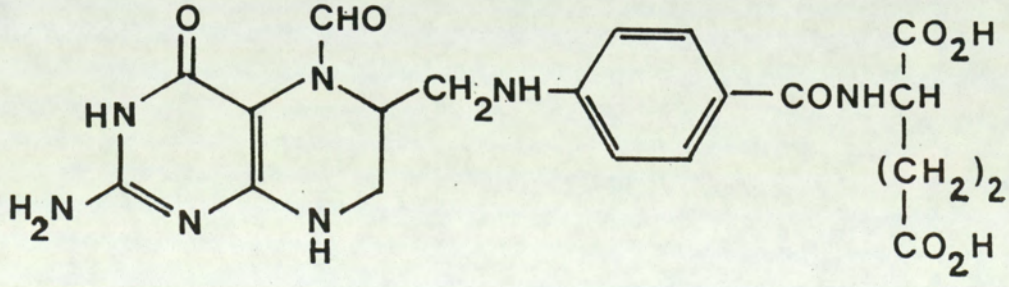
	R_1	R_2
AMINOPTERIN	H	H
METHOTREXATE	Me	H
3',5'-DICHLOROMETHOTREXATE	Me	Cl



FOLIC ACID



FOLINIC ACID



be protected by administration of citrovorum factor* (26,30,31). The protective agent shows limited entry into the CNS thus if this is the target compartment the chemotherapeutic index for lipophilic antifolates can be amplified (32).

Lipophilic antifolates which are derivatives of 2,4-diaminopyrimidine have been studied most extensively and metoprine (Figure 1.2) may be regarded as the prototype. McCormack (33) suggested that replacement of the 3-chloro group by nitro may produce a compound that is a useful alternative to metoprine, because the nitro group may be reduced rapidly in vivo to an amino function, resulting in a less active metabolite.

The present work is concerned with elucidating the pharmacokinetic parameters of MZP, 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine. For most of the work the drug was studied in the form of the ethanesulphonate salt, MZPES (Figure 1.2). MZP bears a closer structural resemblance to etoprine (Figure 1.2) than to metoprine.

Organic azides possess several potentially valuable characteristics which can be exploited in drug design. The aryl azido substituent has a moderate inductive electron-withdrawing effect on the benzene ring, similar to that of the halogens (36), thus MZP should have properties similar to those of metoprine and etoprine. The azido group is very susceptible to both chemical and biological transformations. Aryl azides can undergo photolytic and radiolytic degradation to short-lived highly reactive electrophilic species (nitrenes) capable of versatile interactions with bio-macromolecules (37) and they have been exploited in the "photoaffinity"

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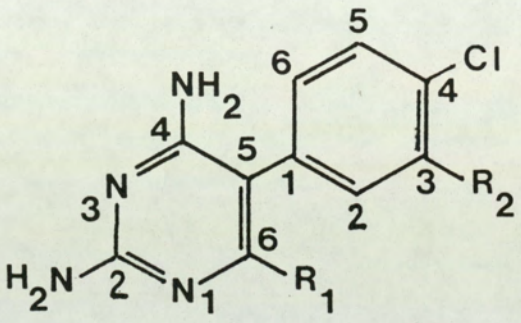
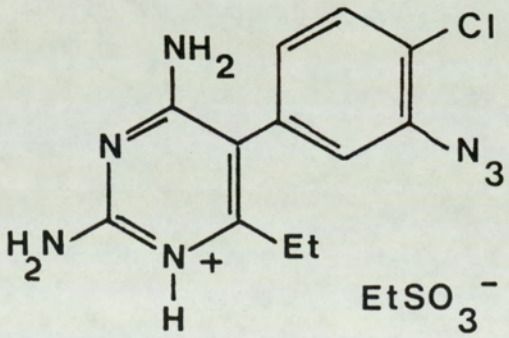
* Citrovorum factor is the Leuconostoc citrovorum growth factor. Folinic acid (leucovorin, Figure 1.1) is the synthetic product. Calcium folinate is the form for therapeutic usage (34) and is half as active as natural citrovorum factor (35).

Figure 1.2 Chemical structures of pyrimethamine, etoprine, metoprine, MZPES, DAMP and DAEP

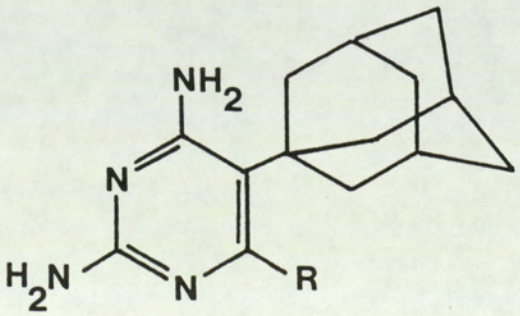
	R_1	R_2
PYRIMETHAMINE	Et	H
ETOPRINE	Et	Cl
METOPRINE	Me	Cl



MZPES



	R
DAMP	Me
DAEP	Et



labelling technique for providing information on the binding and/or catalytic sites in macromolecules (38). The azido group is readily reduced *in vivo* to the amine, which can then be further metabolized by acetylation (39). Introduction of an azido group to pyrimethamine (Figure 1.2) should therefore result in a molecule with a shorter biological half-life than the antimalarial. Reductive degradation of MZP to the corresponding amino compound, 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine (MAP) introduces an alternative basic site into the molecule to afford a polar compound which should be more rapidly excreted. Thus MZP was synthesized in an attempt to produce an antitumour antifolate having activity against cell lines resistant to MTX by virtue of a transport defect, having a short half-life (thus allowing easy assessment of optimum dosage level) and showing minimal toxicity (which could be readily controlled by dosage scheduling). A short half-life relative to that of other lipophilic antifolates may also alleviate problems of cumulative toxicity to normal cells seen with these drugs (20-27).

1.2 Historical Perspective

In the late 1940s Hitchings *et al* (40,41) at the Wellcome Research Laboratories reported that certain 2,4-diaminopyrimidines were powerful competitive antagonists of folic acid (Figure 1.1) in cultures of *Lactobacillus casei*. Many derivatives of 2,4-diaminopyrimidine substituted in the 5 and 6 positions were subsequently synthesized, including pyrimethamine, metoprine and etoprine. Pyrimethamine was found to be the most effective antimalarial in experimental animals (42,43) and was subsequently used for the treatment of malaria in man (44). Derivatives of 2,4-diaminopyrimidine with hydrogen or the smaller alkyl groups in the 6-position and with 5-(3',4'-dihalophenyl) substitution were seen

to have a number of biological activities in common with those of MTX and aminopterin, including activity against mouse sarcoma 180 (45,46). Metoprine and etoprine were subsequently shown to be active against MTX-sensitive and MTX-resistant strains of murine AK4 leukaemia (47) and folinic acid was seen to diminish the toxicity of 2,4-diaminopyrimidines in experimental animals (47,48). Metoprine exhibited activity against murine L1210 leukaemia and synergism between metoprine and MTX was observed (49). Metoprine and etoprine were shown to be active against Ehrlich mouse carcinoma (50).

The demonstrated antitumour activity of metoprine in rodents led to a clinical study of the drug in 1954 (51). Metoprine was administered PO 2.5 to 10 mg in children and 2.5 to 20 mg in adults, in most cases on a daily basis, for maximally 41 days in children and 68 days in adults. Three out of 12 children with acute leukaemia resistant to MTX showed objective responses and 2 of these patients achieved complete responses. Severe toxic manifestations appeared in the gastrointestinal tract, skin and haemopoietic system, but 2 patients who received leucovorin experienced no bone marrow toxicity. The drug was considered to be too toxic for routine use and the trial was abandoned. In 1954 analytical methods to define kinetic parameters of lipophilic antifolates were in their infancy and were not employed in this trial. It is now known that these drugs persist in the body for long periods; De Jager (17) reported a plasma half-life of 150 hours for metoprine in man. In retrospect it is not surprising that metoprine accumulated to toxic levels in patients treated daily with the drug.

In 1961 it was shown that the uptake of MTX by Lactobacillus arabinosus was mediated by an active cellular transport system, whereas the uptake of pyrimethamine was apparently by passive diffusion (52). In 1965 the 2,4-diaminopyrimidines were shown to have the same cytotoxic mode of action as MTX; they strongly bind to and

inhibit the enzyme dihydrofolate reductase (DHFR) (53).

Two events stimulated a renewal of interest in the lipophilic antifolates as anticancer agents. The first was the discovery by Nichol and Mishra, in 1967, that these compounds were active against solid tumours unresponsive to MTX (5,13) and the second was a clinical demonstration of the activity of pyrimethamine against CNS leukaemia (54). The Walker 256 (W-256) carcinoma in rats is a model solid tumour system refractory to MTX by virtue of impaired cellular transport. The tumour was highly sensitive to metoprine however (5,13) and it appeared likely that the superior efficacy of metoprine was due to differences in the permeability characteristics of W-256 cells to the two drugs. In 1971 pyrimethamine produced remissions of 7 and at least 6 months duration in two episodes of meningeal leukaemia in a patient with acute myeloblastic leukaemia. Haematological and gastrointestinal toxicity was readily reversible with folinic acid. The study demonstrated that pyrimethamine crosses the blood-brain barrier. MTX does not penetrate into the CNS in therapeutic amounts when given in tolerable oral or parenteral doses (54).

Metoprine has been evaluated in the clinic since 1975 and has shown limited therapeutic effect against some malignancies refractory to other treatment. Administration has always been by the oral route. Some activity against CNS leukaemia (55), lung cancer (55,56), hypernephroma (56,57), renal cell carcinoma (30), bladder cancer, mycosis fungoides (55), brain gliomas (18), colorectal carcinoma (58), squamous cell carcinoma, melanoma, soft tissue carcinoma (30), epidermoid carcinomas of the head, neck and lung (22,59) and miscellaneous solid tumours (26,60) has been demonstrated. The agent was of no value alone against advanced pancreatic adenocarcinoma (61) or non-small-cell lung cancer (62). The efficacy of metoprine against MTX-resistant tumours in man has not been clearly established. Toxicity in these trials was erratic

and unpredictable and the optimal dose and schedule of administration of metoprine has not yet been defined.

1.3 Drugs related to Metoprine and MZP

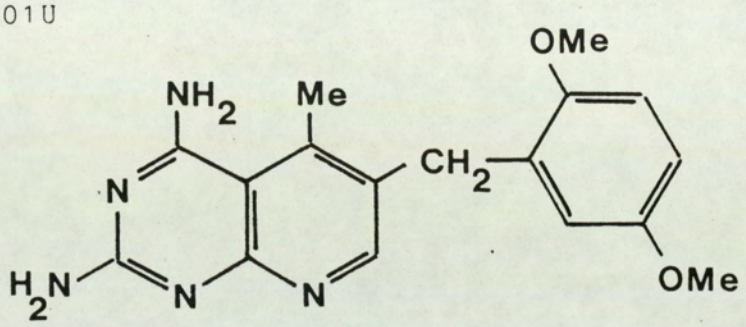
The encouraging antitumour properties of pyrimethamine, etoprine and metoprine led to the synthesis of a series of 2,4-diaminopyrimidines substituted with the lipophilic 1-adamantyl group at position 5 and DAMP and DAEP (63) were studied most extensively for antineoplastic activity. Several other lipophilic antifolates have been shown to possess anticancer properties. BW 301U (Piritrexim, ref 64) is a derivative of 2,4-diaminopyrido [2,3d] pyrimidine. Trimetrexate (65) has some structural similarity to MTX. The formulae of these compounds are shown in Figures 1.2 and 1.3.

Esters and amides of MTX, aminopterin and 3'5'-dichloromethotrexate (Figure 1.1) have been of interest in recent years. Replacement of the negatively-charged carboxylate groups on the glutamate side-chains by lipid-solubilizing hydrocarbon moieties has made these derivatives attractive as a potential means of drug delivery to the CNS and of killing cells resistant to MTX by virtue of a transport defect. Derivatives which have shown promising activity in vitro and in vivo are: alkyl and aralkyl esters of MTX and 3', 5'-dichloromethotrexate (66,67), gamma-tert-butyl esters of MTX and aminopterin (69) and gamma-monoamides of MTX and aminopterin (69).

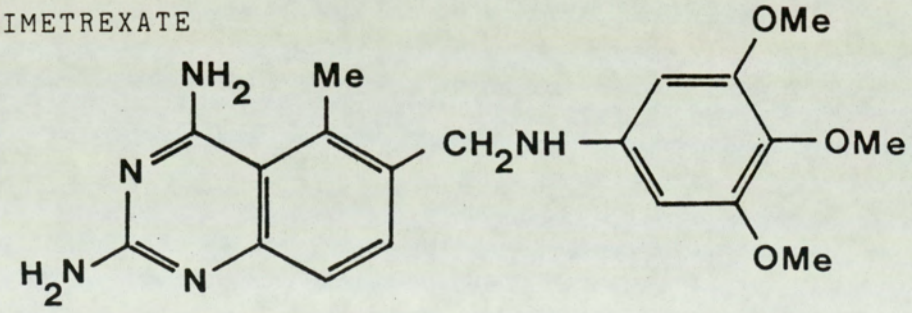
Azidocillin (70), azidoamphenicol (71), azidomorphine and azidocodeine (72) are therapeutic azides which have appeared in the literature. Azidodeoxythymidine (Retrovir) is an antiviral drug of use in the treatment of AIDS (73). An azido analogue of griseofulvin has been patented (74). In contrast to MZP all these drugs are aliphatic azides.

Figure 1.3 Chemical structures of BW 301U and trimetrexate

BW 301U



TRIMETREXATE



1.4 Physicochemical properties of non-classical antifolates

The predominant physicochemical feature of the small-molecule antifolates is their lipophilicity. This property allows these drugs to distribute widely and rapidly into different body compartments and to cross the blood-brain barrier (6). Lipophilicity can be expressed quantitatively as the logarithm of the octanol-water partition coefficient (log P). Table 1.1 lists log P values for the major non-classical antifolates, for MZP and, for comparison, MTX and 3',5-dichloromethotrexate. Etoprine, MZP and metoprine are the most lipid-soluble drugs. Additional lipophilicity is imparted to pyrimethamine (log P 2.69) when an azido group is introduced to the 3'-phenyl position to produce MZP (log P 2.96). MTX is a polar compound and has a negative log P (-1.85) but it is rendered less hydrophilic by the substitution of chlorine atoms into the benzene ring to produce 3',5'-dichloromethotrexate (log P -0.80). Trimetrexate, having a log P value of 0.88, is moderately lipophilic.

Metoprine, pyrimethamine, DAMP, BW 301U and trimetrexate enter cells very rapidly, possibly by a simple diffusion process, and their transport may not be carrier-mediated (7-9,77,79). BW 301U and trimetrexate showed increased uptake and cytotoxic potency over MTX when tested *in vitro* against some rodent tumour cells (7,8). It is the lipophilicity of these molecules that allows facile cell entry, thus MZP should be capable of crossing cell membranes rapidly and extensively. Although BW 301U is less lipophilic than metoprine it was found (8) to be considerably more potent than metoprine in inhibiting growth of some tumour cells *in vitro*, but in another study (9) a positive correlation was found between log P values for DAMP, metoprine and pyrimethamine and the extent of uptake and growth inhibitory potency of these drugs in various cell lines. Such a correlation was also evident between log P values and affinity for DHFR (9). The benefits of lipophilicity are limited however; a drug

Table 1.1 Log P and pK_a values for some antifolate drugs

Drug	Log P	pK_a	Percent ionized at pH 7.4	Reference
Etoprine	3.19	7.20	38.7	75
MZP	2.96	7.19	38.7	76
Metoprine	2.82	7.15	35.9	75
Pyrimethamine	2.69	7.34	46.6	75
DAMP	2.64	--	--	9
BW 301U	1.73	7.1	33.4	77
Trimetrexate	0.88	--	--	78
3',5'-Dichloro-methotrexate	- 0.80	--	--	78
MTX	- 1.85	4.7*	0.5	75

* Determined in 30% methanol in water.

which is too lipophilic may not be available in vivo to inhibit the target enzyme because the drug is bound at other sites (75,80).

The value of partition measurements using water as the polar phase can be questioned. The amount of neutral form of a weak electrolyte available to enter an organic solvent depends upon the pK value of the solute and the pH of the aqueous phase. The Henderson-Hasselbach equation defines the proportions of ionized and neutral species present as a function of pH when a weak base or acid is in solution. If the pH of the solvent is not constant, as is the case with water, the amount of unionized drug present, hence measured log P values, will vary. A buffer of defined pH should therefore be employed when measuring partition of a weak electrolyte.

Table 1.1 also presents pK_a values for some antifolate drugs. The lipophilic compounds have pK_a values which are close to physiological pH (7.4) and they are approximately 30 to 50% ionized in the blood (Table 1.1). In each case adequate concentrations of neutral base, for cell penetration, and N-1 protonated species, for enzyme inhibition (Section 1.5), will be available.

The water solubility of MZP free base is low but MZPES is sufficiently soluble (14 mg/ml) for formulation of the salt as a 1% w/v solution (81).

1.5 Dihydrofolate reductase and its inhibition

DHFR (tetrahydrofolate NADP+ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). The coenzyme THF serves as a carrier of one-carbon fragments in a number of biosynthetic transfer reactions. DHFR is an intracellular target for MTX. As a target site for chemotherapeutic agent DHFR is intimately

involved in the continuous synthesis of thymidylate (dTMP). Thymidylate synthase (EC 2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to dTMP in the presence of N^5 ,N^{10}-methylene THF. The latter compound not only supplies the one-carbon fragment to the 5'-position of the uracil ring of dUMP but also serves as a reductant, supplying a hydrogen from C-6 of the reduced pyrazine ring of THF, yielding DHF. Thus THF must be regenerated via the DHFR- catalyzed reaction in order to maintain the intracellular pool of THF one-carbon derivatives for both dTMP and purine nucleotide biosynthesis. Inhibition of DHFR leads to a deficiency of dTMP and purines and thus to a disruption of nucleic acid synthesis (82,83).

The 2,4-diaminopyrimidine antifolates bind to DHFR in their protonated forms. The active sites of DHFR enzymes are aspartate and glutamate and these anionic residues attract the protonated N-1 of the 2,4-diamino-pyridimines (84). The most active antifolates in this class have pK_a values of 7 or higher (85). The pK_a of MZP is 7.19 (Table 1.1) thus this drug could be expected to bind tightly to the enzyme.

A parameter used to assess antitumour activity of antifolates is their ability to inhibit purified DHFR, but success in finding clinically useful drugs starting with such studies has been limited (80) because of the many variables that will affect a drug when administered to a patient. Enzyme inhibition studies do provide useful comparative data however. The information most relevant to the clinic is the potency of these drugs against human DHFR and such experiments have shown that, on the basis of ID_{50} values, metoprine and etoprine were considerably more potent than pyrimethamine against DHFR from human erythrocytes and from human acute lymphocytic leukaemia cells, but the potency of MTX exceeded that of the lipophilic antifolates (6). Trimetrexate approached the potency of MTX against acute lymphocytic leukaemia enzyme and against DHFR from MTX-resistant L1210 cells (7). The

inhibitory potency of BW 301U against DHFR from human chronic granulocytic leukaemia cells was 20-fold that of metoprine and marginally exceeded the potency of MTX (8). DAMP was more potent than pyrimethamine against DHFR from sarcoma 180 cells but showed affinity for the enzyme 100 times lower than that of MTX (86).

The inhibitory potency of MZP exceeded that of pyrimethamine against rat liver DHFR (ID_{50} s 1.3 and 1.8nM respectively, ref 87). A DHFR inhibition constant of the order of 1nM is desirable and MZP had Ki values of 1.6nM against rat liver DHFR and 2.4nM against L1210 DHFR (87). MAP was 50-fold less active than MZP as a DHFR inhibitor (88).

Browman (89) suggested that the only locus of cytotoxic action of metoprine is DHFR, but other workers (86, 90-93) have claimed that metoprine, DAMP and trimetrexate have sites of cytocidal action supplementary to DHFR. DAMP exhibited some activity against thymidylate synthase (94) but the possible additional sites of action of lipophilic antifolates have not yet been fully described. BW 301U appeared to be a selective inhibitor of DHFR (8).

1.6 Cell culture studies

Cellular resistance to antimetabolites can be natural or acquired and may result from a variety of events. Cell membrane transport may be impaired with a consequent decrease in the amount of drug entering the cell. 'Gene amplified' cells are those in which there is an increased amount of target enzyme. Structural alteration in the active site of the enzyme may lead to altered enzyme-inhibitor kinetics and reduced affinity of the drug for the enzyme (95,96).

Cell culture studies have demonstrated that metoprine and BW 301U inhibited the growth of a cell line resistant to MTX by virtue of a transport defect (15) and that

metoprine, BW 301U and trimetrexate were more potent than MTX against some cell lines showing both MTX-uptake defects and overproduction of DHFR (12,15). Low concentrations of metoprine relative to MTX inhibited deoxyuridine incorporation into the DNA of cells having high levels of drug resistant DHFR (96) and BW 301U was more potent than MTX in inhibiting deoxyuridine incorporation into the DNA of a MTX transport-deficient/DHFR overproducing cell line (97,98). Thus lipophilic antifolates can penetrate and kill cells resistant to MTX because of transport impairment and may circumvent MTX-resistance caused by amplification of DHFR. The observed biological differences between MTX and the lipophilic drugs may be due to the fact that the non-classical antifolates enter cells rapidly, possibly by simple diffusion and, unlike MTX, they may not be dependent on active transport (7-9,77,79). The precise transport mechanism of lipophilic antifolates is still uncertain however. A study (99) on a cell line sensitive to MTX but resistant to trimetrexate, metoprine, BW 301U and gamma-tert-butyl MTX ester indicated that lipophilic antifolates may enter cells by a process more complex than simple diffusion. Resistance to these drugs developed possibly because of deletion of a specific transport system or alteration in the cell membrane structure. Furthermore, ability of a drug to enter a cell and interact with a target enzyme is no guarantee that it will exert a cytotoxic effect (95). A subline of an L1210 parent cell sensitive to metoprine and MTX was resistant to metoprine but not to MTX. DHFR activity and total accumulation of metoprine were approximately equal in both parent and subline. The K_i for DHFR from the subline was increased 3.0-fold for MTX and 3.5-fold for metoprine relative to that from the parent line. Pleiotropically drug-resistant cells insensitive to adriamycin, vinca alkaloids, trimetrexate and BW 301U, but sensitive to MTX, gamma-tert-butyl MTX ester and metoprine have recently been described (100).

A cell subline with acquired transport-resistance to MTX may be more sensitive than the parent line to lipophilic

antifolates (11,14). 5-methyltetrahydrofolate, the major circulating folate in blood, shares the MTX-route for cell entry. Thus impaired MTX transport in such a mutant will be accompanied by lowered uptake of 5-methyltetrahydrofolate, decreasing the ability of the cell to sustain tetrahydrofolate-dependent reactions in the face of a DHFR blockade induced by lipophilic antifolates. Because folinic acid is also transported by the MTX-carrier, in cells with impaired MTX transport the efficacy of folinic acid protection may be decreased, resulting in selective protection of normal cells from lipophilic antifolates (11,14).

MZPES was designed to overcome the deficiency in MTX transport exhibited by some cells and has shown activity against such a cell line (L5178Y murine leukaemia, ref 88).

1.7 Recent experimental anti-tumour studies

Studies concerned with evaluating the experimental antitumour activity of lipophilic antifolates in animals centre around three main points. The first of these is assessment of their activity against a range of experimental tumours, including those resistant to MTX. The second is evaluation of the ability of these drugs to penetrate into the CNS. The third is determination of the optimal dosage schedule of drug and folinic acid so as to provide a safe but effective combination.

In 1952 Burchenal (47) demonstrated that metoprine and etoprine were active against MTX-resistant murine AK4 leukaemia and in 1967 Mishra, Rosen and Nichol (5,13) showed that the rat W-256 carcinoma was sensitive to metoprine. The lipophilic antifolates show similarities but also striking differences in their inhibitory activities. Thus BW 301U and DAMP caused regression of W-256 carcinoma but were active and inactive respectively against sarcoma 180 in mice (8,101). Both drugs showed little activity against L1210 leukaemia in mice (8,101) but trimetrexate

was active against this neoplasm (7). A potentially useful additive effect of trimetrexate and MTX was observed in mice bearing L1210 leukaemia (102). Against murine B16 melanoma BW 301U was virtually inactive but trimetrexate was active (7,8). Such diverse effects may be due to differences in the cellular transport of these compounds.

MZP has been evaluated for its activity against a range of transplantable murine tumours. These tests were carried out according to the standard protocols advocated by the National Cancer Institute of the USA (103,104). Two types of assay were employed; the survival time assay, used in tests against systemic cancers (such as L1210 and P388 leukaemias), where results were assessed in terms of the increased survival of the test (T) mice in comparison with the control (C) batch, and the tumour inhibition assay used for solid tumours (such as the colon 38 adenocarcinoma), where results were assessed in terms of a measurable tumour inhibition. Table 1.2 summarizes the results and compares them with those obtained for metoprine and trimetrexate monoacetate using the NCI activity criteria. MZP is active against the P388 and L1210 leukaemias, the B16 melanoma, TLX5 lymphoma and the MTX transport-resistant reticulum cell sarcoma M5076. The drug is inactive against the colon 38 adenocarcinoma, the Lewis lung carcinoma and $CD8F_1$ mammary tumour. Its activity is favourable in comparison with metoprine and approaches that of trimetrexate. The demonstrated activity of MZP against the murine models was sufficient to initiate a pre-clinical programme leading to the phase I trial of the drug.

Metoprine and etoprine have shown activity against ethylnitrosourea-induced brain tumours in rats (106). This study provided evidence that these drugs penetrate into the CNS and that they are potentially useful for the treatment of brain neoplasms in man, but using the avian sarcoma virus induced rat glioma model less encouraging results were obtained (35). DHFR is present in both human gliomas and rat gliomas induced with avian sarcoma virus, but is

Table 1.2 Activity of MZP, metoprine and trimetrexate against experimental transplantable murine tumours

Tumour	Optimum T/C (%) MZP	Activity rating MZP	Metoprine	Trimetrexate*
P388	151	+	+	++
L1210	158	++	+	++
B16	157	++	-	++
TLX5	135	+	-	n/d
M5076	n/d	++	n/d	n/d
Colon 38	n/d	-	n/d	+
Lewis lung	-	-	-	-
$CD8F_1$	-	-	-	++
Reference	88, 105	88, 105	88, 105	25

T - survival of test mice C - survival of control mice
* Monoacetate salt
n/d - not determined

not detectable in normal adult brain, making it an appropriate enzyme to inhibit when attempting to destroy only the tumour cells present in the brain. Metoprine reached high levels in both normal and gliomatous rat brain tissue after IP injection, but there was no increase in survival of treated rats over controls. The lack of effectiveness was probably due to the refractory nature of the tumour rather than inadequate exposure to the drug.

Determination of optimum dosage schedules of metoprine in experimental animals has proved to be difficult. The drug showed appreciable activity against ascitic, solid and intracranial sarcoma 180 in mice, but antitumour effects were highly schedule and dose-dependent within the range 8 to 40 mg/kg. With citrovorum factor rescue 24 hours after

metoprine (8 x 16 mg/kg) the ILS of mice bearing ascitic tumour was 158% (107,108).

1.8 Toxicology of lipophilic antifolates

The lipophilic 2,4-diaminopyrimidines show two distinct types of toxicity in mammals. At doses below the LD_{50} delayed haematological and gastrointestinal effects are seen. These changes are due to the antifolate properties of the drugs and can be ameliorated by the administration of citrovorum factor (109-111). Higher doses cause acute convulsive deaths. These deaths are not characteristic of folic acid deficiency and can be prevented by barbiturates but not by folinic acid (110).

Bone marrow was suppressed less by the adamantyl antifolates than by metoprine but DAMP and DAEP were considerably more acutely neurotoxic (19). The latter effect may be due to the lipophilic adamantyl groups which allow for rapid penetration of the blood-brain barrier. The relationship of metoprine to DAMP in this respect cannot be related to log Ps however, because metoprine has the higher value (2.82 and 2.64 respectively, Table 1.1).

Folate-related and CNS toxicity of metoprine has been observed in man (20-24,26,51,55,56,60,112,113). The former effects could be controlled by giving folinic acid concurrently (folinic acid protection) (20,26,30,56,59,60,112) or subsequent to administration of metoprine (folinic acid rescue) (31,56,59). The use of folinic acid together with a drug having such a long half-life (216 hours, Table 1.5) presents an unusual problem in determining the optimal dosage regimen of both agents and the superiority of either mode of administration of folinic acid has not been clearly demonstrated. Skin reactions have been reported following administration of metoprine (20,55,112), BW 301U (114-116) and trimetrexate (27,117-120) to man. Bone marrow suppression and gastrointestinal disturbances have been

observed in phase I clinical trials of trimetrexate (117-122). Haematological and mucosal sequalae were evident in phase I trials of BW 301U (114-116).

The CNS, gastrointestinal and cutaneous toxicities of metoprine and trimetrexate may involve inhibition of histamine metabolism (28,29). In the CNS histamine is metabolized primarily in a reaction catalyzed by histamine N-methyltransferase (HMT), whereas in many tissues other than brain histamine can be disposed of by diamine oxidase. Some lipophilic antifolates have been evaluated as inhibitors of these enzymes and against HMT isolated from bovine brain the order of potency was trimetrexate >DAMP> metoprine. BW 301U had weak activity as an inhibitor of HMT. Trimetrexate and DAMP were the most potent inhibitors of diamine oxidase from rat caecum, whereas metoprine and BW 301U were relatively weak inhibitors of this enzyme. BW 301U appears to have a potential advantage over metoprine and trimetrexate by virtue of its significantly lower inhibition of histamine metabolism, which should reduce the risk of histamine-related side effects. MZP is a weak inhibitor of HMT (88).

Table 1.3 presents acute single-dose toxicity data for metoprine, MZPES, DAMP, DAMP ethanesulphonate salt (DAMPES) and trimetrexate in the mouse. MZPES is considerably less toxic than metoprine (LD_{50} IP 75 and 38 mg/kg respectively) and is apparently less toxic than DAMPES (LD_{50} 30 mg/kg). The route of administration of DAMPES was not published. MZPES and trimetrexate are approximately equitoxic in the mouse. MZPES is considerably less toxic by the IP route than by the IV route (LD_{50} 75 and 44 mg/kg respectively).

Table 1.3 Mouse acute single-dose toxicity of some lipophilic antifolates

Drug	Route	LD_{10} (mg/kg)	LD_{50} (mg/kg)	Reference
MZPES	IP	57(44-70)	75(63-88)	123
	IV	18(9-27)	44(29-59)	123
Metoprine	IP	-	38	123
	SC	-	50-55*	107
DAMP	n/s	-	50-100	101
DAMPES	n/s	-	20-40	101
Trimetrexate	IV	55	62	124

95% confidence limits in parentheses

* administered as hydrochloride salt but LD_{50} expressed as free base

n/s - not stated

1.9 Analysis of lipophilic antifolates

Monitoring of levels of anticancer drugs in body fluids is desirable to define the margins which exist between efficacy and adverse effects (125). Assay of a drug in tumour tissue may be needed to obtain evidence that it reached target cells (32). Sensitive methods of analysis are required for lipophilic antifolates because of the small doses given and their long half-lives, which result in very low levels of these drugs being present in fluids and tissues for extended periods after administration.

Methods of analysis based on the binding of antifolates to DHFR have been described. The addition of a DHFR inhibitor to an *in vitro* DHFR-NADPH-DHF system leads to a reduction in the rate of production of THF which is related to the amount of inhibitor present. This observation has been used to assay metoprine (126) and

trimetrexate (16,119,127,128) in biological samples. Trimetrexate and ^{3}H MTX compete for binding to DHFR and this phenomenon has been used to assay trimetrexate in whole plasma, urine and CSF (129). After binding was complete free ^{3}H MTX was separated from bound ^{3}H MTX by charcoal adsorption and a standard curve of trimetrexate concentration versus amount of ^{3}H MTX bound to DHFR was prepared. Trimetrexate concentrations of 0.0007 mg/L could be measured in plasma with CVs of less than 10%. Because there were no separation steps in these procedures any DHFR inhibitors would affect the assays. Drug metabolites may preserve a high degree of binding affinity for DHFR and, if so, drug levels measured will be falsely high. Such metabolites may be active antitumour entities and will be important to measure along with the parent compound. For specificity however a prior separation of such metabolites is essential.

Pyrimethamine blocks the utilization of folic acid by Streptococcus faecalis and inhibition of the growth of cultures of this organism has been used to assay the drug in biological material (54,130-132). This procedure suffers from the imprecision associated with biological assays.

Chemical and physicochemical methods for analysis of lipophilic antifolates normally require an initial extraction of the drug from basified sample into an immiscible solvent and 1,2-dichloroethane has been widely used (6,75, 133-139). Other organic solvents that have been used are dichloromethane (140-142), toluene (143), ethyl acetate (17,144,145), diethyl ether (146-148) and 1-chlorobutane: dichloromethane 96:4v/v (149). Diethyl ether:ethanol 3:1 v/v was used to extract pyrimethamine, metoprine and their metabolites from rat urine. Prior saturation of samples with ammonium sulphate was necessary (150). Dichloromethane extracts of plasma were freed from endogenous materials by passage through silica gel cartridges, thus allowing the analysis of BW 301U by TLC without interference (141). Plasma samples containing trimetrexate were applied to C18

sample preparation cartridges, which were washed to remove unwanted endogenous materials before elution of the drug and analysis by GC (151) or HPLC (16,119,152). Plasma or brain containing metoprine was mixed with methanol: phosphate (0.02M, pH 7.5) 40:60 v/v, a reagent that precipitates endogenous material, and the supernatant liquid was analysed by HPLC (153).

Pyrimethamine has been measured in biological samples by solvent extraction, back-extraction into acid then UV spectrophotometry(130,131,146,154,155) and, after solvent extraction, by its colour reaction with methyl orange (130,154). These methods were tedious, insensitive and required corrections for blank values. The users generally recognized the limited ability of the procedures to distinguish between intact drug and metabolites.

Radiochemical methods of analysis have been widely used in lipophilic antifolate pharmacokinetic studies. The 2,4-diaminopyrimidines can be labelled with ^{14}C at position 2 of the pyrimidine ring (6,19,75,155,156). Trimetrexate also may be labelled with ^{14}C (127). The 2,4-diaminopyrimidines have been counted directly in biological media (155). ^{14}C labelled pyrimethamine, metoprine and etoprine have been measured in tissues, urine and plasma by extraction, TLC and autoradiography of spots (75,150). Liquid scintillation counting has been used to measure radiolabelled metoprine, etoprine (6,75), pyrimethamine (6,32,156) and trimetrexate (128,157) directly in biological fluids and tissues and radiolabelled DAMP has been measured by this method after extraction (19). Those methods of analysis which do not include a prior separation step are non-specific and may include metabolite levels in observed drug concentrations.

Column chromatography has found some application in this field. DAMP was separated from its metabolite by passage of the supernatant from plasma or tissue homogenates through a Sephadex G-25 column (19). Extracts of rat urine containing pyrimethamine or metoprine and their

metabolites were fractionated on a fluorisil column before isolation of individual metabolites by preparative TLC (150).

Quantitative TLC has been used to measure pyrimethamine (6,75,133,134,137,139), metoprine, etoprine (6,75, 134,139), DAMP (19) and BW 301U (141) in biological samples. Two-dimensional TLC has been used to identify and quantify the metabolites of pyrimethamine and metoprine (150). Detection and quantitation in TLC has been by natural fluorescence (137,141), fluorescence induced by spraying the plate with a solution of ammonium hydrogen sulphate and drying (139), UV absorption (6,133,134) and autoradiography of $2^{14}C$ labelled compounds (75,150). Liquid scintillation counting has been used to measure radiolabelled lipophilic antifolates extracted from adsorbent (19,150). Taking 1 ml of plasma and using fluorescence detection as little as 0.005 mg/L of BW 301U could be measured (141). Freedom from interference by co-extracted endogenous substances (133,141) and by dapsone (133) has been claimed, as has specificity of measurement of intact drugs in the presence of metabolites (75). However, this group (75) did not publish proof that the method they described separated pyrimethamine, metoprine and etoprine from their respective metabolites.

GC has been employed to measure pyrimethamine in plasma and urine (142,143,147,158) and for assaying metoprine in plasma, urine, cerebrospinal fluid and brain tissue (17,145). Stationary phases were OV 17 or OV 101 and electron-capture or nitrogen detectors were used. Trimetrexate has been extracted from plasma, trimethylsilylated, chromatographed on a cross-linked methyl silicone liquid phase and detected by MS with selected-ion monitoring (151). Taking 1 ml samples the sensitivity limit of GC was generally about 0.005 mg/L. Specificity in the presence of MTX, 3',5'-dichloromethotrexate and aminopterin (151) has been claimed. GC is unsuitable for analysis of N-oxides because these compounds revert to their desoxy analogues at elevated temperatures (159,160).

HPLC has been used to measure pyrimethamine (135,136, 138,140,148,149) pyrimethamine and, simultaneously, its N-3 oxide (144), metoprine (153), BW 301U (141) and trimetrexate (16,119,128,152). Although normal-phase HPLC, using silica as adsorbent, was satisfactory for analysis of pyrimethamine (138,149) this mode was unsuitable for BW 301U because of the strong affinity of the drug for silica which resulted in asymmetric peaks and excessive capacity factors (141). Biotransformation of a drug may result in a diversity of metabolites with widely differing physicochemical properties. Adsorption HPLC is generally limited to analysis of metabolites whose physicochemical properties are similar to those of the parent drug, whereas reversed-phase HPLC is suitable for compounds with widely-ranging pK values and lipophilicities (161). Furthermore, polar compounds are generally not retained on reversed-phase columns and will not interfere with the analysis of more lipophilic drugs (162). Thus reversed-phase HPLC is eminently suitable for analysis of lipophilic antifolates and their metabolites and has found wide application in this field. C_1, C_8 and C_{18} alkyl bonded phases have been used. Sample extracts are normally injected into columns but Weir et al (128) mixed samples containing trimetrexate with mobile phase and injected the homogenates. Most workers have employed isocratic elution but gradient elution was used (16) for analysis of trimetrexate in plasma. Ion-pairing reagents, for instance, octanesulphonic acid (144), have been employed in reversed-phase HPLC. Strongly-absorbing chromophores in the 240 to 280 nm region have allowed lipophilic antifolates to be detected by UV spectroscopy. Timm and Weidekamm (149) found the fluorescence of pyrimethamine to be more specific than the absorbance, but weaker. Trimetrexate has been detected electrochemically (119,128). Recovered fractions have been assayed by a DHFR inhibition procedure and, in the case of ^{14}C labelled trimetrexate, by scintillation counting (16).

The limit of detection of lipophilic antifolates by

HPLC is typically 0.005 mg/L, taking 1 ml of sample. HPLC procedures may be specific for lipophilic antifolates in the presence of co-administered drugs (138,140) and drug metabolites (152).

NMR and MS have been used to confirm the structures of some metabolites of pyrimethamine, metoprine (150) and trimetrexate (163).

1.10 Pharmacokinetics and metabolism of lipophilic antifolates

1.10.1 Gastrointestinal absorption and bioavailability

Studies (154,155) using monkeys demonstrated that absorption of pyrimethamine from the gastrointestinal tract was slow, but regular and virtually complete. In cancer patients treated with metoprine PO maximum plasma concentrations were reached about 15 hours after single doses of 90 mg/m² and about 48 hours after 120 to 180 mg/m². Peak concentrations were not strictly dose-related (17). This may reflect variable absorption, distribution, metabolism or excretion.

After ingestion of trimetrexate (2 mg/kg) by dogs peak plasma concentrations were reached at about 100 minutes and the oral bioavailability was 80% (128). The bioavailability of trimetrexate in monkeys was only 23% however (16). In the former work the drug was measured by a DHFR inhibition assay, whereas in the latter study a more specific HPLC procedure was also employed. Following oral administration to monkeys unchanged trimetrexate accounted for only about 20% of the DHFR-inhibitory activity in plasma, whereas after IV dosage unchanged trimetrexate accounted for 50% of plasma DHFR-inhibitory activity (16). Extensive metabolism to DHFR-inhibitory metabolites following an oral dose accounts for the discrepancy between published F values for this drug. Trimetrexate probably undergoes presystemic metabolism in the intestinal mucosa or first-pass metabolism in the liver (16) and this proposition

Table 1.4 Plasma protein binding of some antifolate drugs

Drug	Concentration (mg/L)	Binding (%)	Reference
Etoprine	1	97	75
BW 301U	n/s	94	115
Trimetrexate	2	90	16
Metoprine	1	88	75
Pyrimethamine	1	87	75
MTX	10	53	75

n/s - not stated

has been confirmed in a study on man (164). The oral bioavailability of BW 301U in the dog was 34% (77).

1.10.2 Plasma protein binding

The lipophilic antifolates bind extensively to plasma proteins (Table 1.4). Etoprine shows a higher affinity than BW 301U, trimetrexate, metoprine or pyrimethamine for these proteins but all 5 drugs bind by 87% or more. This extensive binding contrasts with the lower binding of MTX (53%). It is the free drug fraction that is available to equilibrate with receptor sites (165) and the high affinity of the lipophilic antifolates for plasma proteins could be a disadvantage in restricting the proportion of unbound drug and limiting penetration to target sites. Binding to proteins may however faciliate the accumulation of a drug in cells.

1.10.3 Tissue distribution

Following administration of metoprine (2.8 mg/kg PO) to rats peak tissue/plasma concentration ratios were reached after 5 hours. Ratios were: pancreas 5.3; brain 6.5; lung 30.8. Metoprine had a longer persistence in tissues

than etoprine or pyrimethamine and, in contrast to the other 2,4-diaminopyrimidines, even after substantial depletion the material remaining in the tissues was mostly intact drug (6,75). This difference may be explained by biotransformation of the ethyl substituent in etoprine and pyrimethamine; this group was the site of extensive metabolism of pyrimethamine in the rat (150). Even though etoprine has a greater lipophilicity (Table 1.1) and affinity for proteins (Table 1.4) than metoprine it had lower tissue/plasma concentration ratios (75) and in part this may be due to a faster rate of metabolism for etoprine.

The finding that metoprine had a widespread distribution in tissues has been substantiated in man (75). Autopsy samples taken from a cancer patient who had received metoprine showed highest levels of drug in lung, and substantial levels in lymph node tumour, liver, skin, adrenal gland and brain.

Two hours after administration of BW 301U to the rat tissue/plasma drug concentration ratios were: W-256 tumour 1; lung 3.5; liver 7.0 (77). Maximal tissue concentrations of DAMP were reached in rats 0.33 hours after IV administration of the ethanesulphonate salt. Tissue/plasma ratios were: liver 5.8; pancreas 8.4; spleen 8.9; brain 10.9; kidney 12.9 (19). Trimetrexate reached highest levels in the liver and kidney, and lowest levels in the brain and lung, of dogs (128).

Metoprine penetrated into brain tumours in rats (106) and in man the brain tumour/plasma metoprine concentration ratio was about 4.5 twenty four hours after oral administration (17). In experimental animals DAMP rapidly crossed the blood-brain barrier (19) but BW 301U and trimetrexate did not readily penetrate into the CNS (77,128). This may be explained in part by the relatively low lipophilities of BW 301U and trimetrexate (Table 1.1).

Over the first 48 hours after administration of metoprine to rats the mean CSF/plasma drug concentration ratio was 0.2 (6,75). The lower level found in CSF may be related

to the lower protein content of this fluid in comparison with plasma. Pyrimethamine had a higher CSF/plasma concentration ratio than metoprine or etoprine in the rat (6,75) and is the least bound to plasma proteins (Table 1.4). Thus only unbound drug would appear to enter the CSF. The CSF-plasma relationship probably also depends on lipophilicity. Pyrimethamine is more polar than metoprine or etoprine (Table 1.1) thus may be more soluble in CSF. Trimetrexate entered the CSF of monkeys (16) and dogs (128) after IV administration but CSF/plasma ratios were only about 0.035.

In the rat the uptake of pyrimethamine was most substantial in the liver (166). The extent of pyrimethamine localization in the liver was emphasized in a further study (156) using the isolated perfused rat liver preparation. Following systemic bolus administration of radiolabelled pyrimethamine over 50% of radioactivity left the perfusate and localized in the liver within 30 minutes. After 5 hours 23% of the radioactivity, consisting of unchanged drug with traces of the N-3 oxide metabolite, remained in the liver. During 5 hours 29% of the radioactivity administered was eliminated in the bile.

The disposition of pyrimethamine in the pig has been described (158) as a 3-compartment open model. The faster peripheral compartment probably included the soft organs, such as liver and muscle. In man the pharmacokinetics of pyrimethamine have been described as single-compartment (130,131,167) and 2-compartment (132,149).

1.10.4 Metabolism

Hubbell et al (150) characterized many of the metabolites which appeared in the urine and faeces of rats dosed with radiolabelled metoprine or pyrimethamine (1 mg/kg PO). Recovery of radioactivity was almost quantitativeover 7 days, with the higher proportion found in urine in each case. N-oxides were prepared for comparative purposes by m-chloroperoxybenzoic acid oxidation (168) of the antifolates and

samples were screened by TLC for the presence of these metabolites before and after treatment with titanous chloride, a reagent that reduces N-oxides to their parent compounds (169).

At least 10 metabolites of metoprine appeared in the urine. The N-1 oxide accounted for 60% of the administered dose. The urine also contained small amounts of the glucuronide conjugate of the N-1 oxide and free metoprine. At least 16 metabolites of pyrimethamine appeared in the urine. The N-3 oxide hydroxylated at the alpha-position of the ethyl side chain accounted for 34% of the dose. The urine also contained free pyrimethamine, its N-1 and N-3 oxides, 6-ethyl alpha-hydroxlated pyrimethamine and at least 6 glucuronide conjugates. Pyrimethamine, its N-3 oxide and its alpha-hydroxylated derivative appeared in the faeces. N-oxidation accounted for at least 40% of the administered dose of pyrimethamine.

Hubbell's work showed that oxidation of the ring nitrogens of 2,4-diaminopyrimidines is a major metabolic pathway. Pyrimethamine is N-oxidized mainly at the 3-position, probably because of steric hindrance from the 6-ethyl, or alpha-hydroxylated 6-ethyl, groups. The 6-methyl group of metoprine is less of a hindrance and N-oxidation of this drug is mainly at the N-1 position. The methyl group in metoprine does not appear to undergo biotransformation.

The work described so far enables the probable metabolic sites in MZP to be foretold. Central to such predictions however is the behaviour of the azido group *in vivo*. Azidosulphanilamide has been administered to rats and its metabolism investigated (39). Examination by TLC of the dichloromethane extract of urine of treated rats revealed sulphanilamide, its derivative acetylated on the anilino nitrogen and unchanged azidosulphanilamide. Thus the azido group is susceptible to biological reduction to the amine, which can then be further metabolized by acetylation. Reduction of MZP to MAP and subsequent acetylation of the amino function (to produce MAP-Ac) is therefore to

be expected. N-oxidation of MZP at position 3 should be more extensive than at position 1 because of steric hindrance from the 6-ethyl group. Hydroxylation of the alpha position on the 6-ethyl side-chain may occur. Several of these reactions may take place within individual molecules; the N-3 oxide of MAP, or 6-ethyl alpha-hydroxylated MAP-Ac, may be produced in vivo. Glucuronide conjugates of MZP or its phase I metabolites are to be expected; glucuronic acid can conjugate with a wide variety of functional groups (170) but glucuronidation of the primary amino nitrogens would appear to be most probable in the case of the 2,4-diaminopyrimidines. Other, purely speculative, phase I metabolic reactions of MZP are N-oxidation of the primary amino groups on the pyrimidine ring, oxidation of the azido group to a nitro function, oxidation of the 6-ethyl side chain to a ketone or carboxylic acid, aromatic hydroxylation, acetylation of the primary amino groups on the pyrimidine ring and deazidation of MZP to pyrimethamine. Conjectural phase II reactions are N-methylation and conjugation with sulphate.

The metabolism of trimetrexate has been studied using the isolated perfused rat liver preparation (171). Two metabolites, both capable of inhibiting DHFR, appeared in the perfusate at low levels and in the bile at higher concentrations. DHFR-inhibitory metabolites of trimetrexate were detected on the plasma (119,120) and urine (119,164) of man. A major step in the degradative metabolism of trimetrexate was demethylation of the trimethoxyaniline ring (16,157,163,164) and a prominent metabolite was conjugated with glucuronic acid (163).

1.10.5 Plasma elimination

A species comparison of plasma elimination half-lives of the major non-classical antifolates is presented in Table 1.5. The half-life of MTX in man is also included. Metoprine, etoprine, DAMP and pyrimethamine persist in the blood for long periods, with half-lives ranging from 8 to 16 hours in the rat. BW 301U has a short-life in the rat (0.38 hr).

Table 1.5 Species comparison of plasma elimination half-lives of some antifolate drugs

	Plasma elimination $t_{\frac{1}{2}}$s (hours)			
Drug	Mouse	Rat	Dog	Man
Metoprine	19 (175)	16 (75)	38 (75)	216 (75)
Etoprine	14 (75)	11 (75)	30 (75)	176 (75)
DAMP	--	9 (19)	--	--
Pyrimethamine	6.5 (75)	8 (75)	23 (75)	85 (75)
MTX	--	--	--	30 (172)
Trimetrexate	0.83(127)	--	3.5 (128)	16.4 (119)
BW 301U	--	0.38(77)	7 (77)	4.5 (115)

References in parentheses

Metoprine, etoprine and pyrimethamine bind extensively to plasma proteins (Table 1.4) and they probably also bind to lipids and lipoproteins in tissues, from which they are gradually released into the circulation (6,75). This tissue binding may account for their long half-lives. The half-lives of these drugs are approximately ten-fold longer in man than in the mouse and this may be explained partially by the higher proportion of body fat found in man. Although etoprine has a greater lipophilicity (Table 1.1) and affinity for plasma proteins (Table 1.4) than metoprine its rate of elimination was faster in all species. In part this may be due to etoprine having a faster rate of metabolism. Etoprine possesses an ethyl group, as does pyrimethamine, and a study (150) of the antimalarial in the rat showed that this substituent is the site of extensive metabolism. Although etoprine is a more potent inhibitor of DHFR than metoprine (6) it does not reach so high a tissue concentration (75) and its half-life is not much shorter, thus it does not have any pharmacokinetic advantages over metoprine. The MZP molecule also

contains an ethyl group thus this feature should contribute to rapid plasma elimination of the drug.

Many workers (6,75,125,130-133,138,142,148,149,167, 173) have estimated the plasma elimination half-life of pyrimethamine in man. Mean half-lives were broadly consistent, from 80 to 118 hours, but wide inter-individual variations have been reported. The concentration-time profile in saliva closely reflected that of the unbound drug in plasma (173) thus it is possible to derive the plasma elimination rate constant for pyrimethamine from salivary measurements. Reported plasma elimination half-lives for metoprine in man are 150 (17), 180 (6), 216 (75), and 240 (55) hours. As with pyrimethamine, variation between individuals was large, for example (17) a range of 106 to 183 hours within a group of nine cancer patients given doses from 90 to 280 mg/m². This may be a result of variable absorbtion, distribution, metabolism or excretion.

The plasma disappearance of MTX in man was triphasic, with half-lives of less than 1 hour, 3 to 4 hours and 23 to 31 hours (172). Following 1 hour IV infusion of trimetrexate (10-120 mg/m²) elimination of the drug from human plasma was biphasic ($t_{\frac{1}{2}}$s 0.71 and 16.4 hr) or triphasic ($t_{\frac{1}{2}}$s 0.19, 2.83 and 16.4 hr) (119). The plasma elimination half-life of BW 301U in man was 4.5 hours (115).

The plasma decay curves for pyrimethamine, metoprine and etoprine in man and the dog were sinusoidal, which suggested that these drugs undergo biliary recycling (75). It is likely that the N-oxides of the 2,4-diaminopyrimidines can be reduced to the parent drugs and re-cycled in vivo and this process may be a factor causing the persistence of the drugs in the body (150). , Trimetrexate may be eliminated in the bile then re-absorbed; the drug attained a high concentration in the bile of dogs and the plasma concentration-time curve showed an apparent enterohepatic reabsorption phase (128).

1.10.6 Excretion

Pyrimethamine was excreted in the urine and faeces of mice primarily,as biotransformation products and excretion was prolonged, for up to 7 days (174). In man an oral dose (25 mg) of pyrimethamine could be detected in urine after 16 days (142). Small amounts of pyrimethamine are excreted in the milk of lactating women treated with the drug (175). The excretion of metoprine was slow in man. In cancer patients treated orally at doses of 90 to 280 mg/m² the cumulative 5 day urinary excretion of intact drug was 1.7 to 10% of the dose (n=6) and small amounts were still being eliminated on the tenth day (17). Metoprine was extensively metabolized before excretion from rats (150). Trimetrexate (16,118,128,171) and DAMP (19) were excreted slowly in the urine and faeces of experimental animals and man both as unchanged drugs and metabolites.

1.11 Aims and scope of the present study

The DHFR-inhibitory activity of MZP, its ability to inhibit growth of some transport-deficient MTX-refractory cell lines, its broad spectrum of activity against transplantable murine tumours and its low toxicity in the mouse relative to that of metoprine encouraged further evaluation of the agent for treatment of neoplasms in man.

A prerequisite to clinical assessment is some knowledge of the pharmacokinetics and metabolism of a drug and in the case of MZP these properties may be partially foretold from published studies of other low molecular weight antifolates and azidosulphanilamide. MZP should be readily absorbed from the gastrointestinal tract and may undergo first-past biotransformation in the liver. Anticipated metabolic reactions are reduction of the azido group to the amine, subsequent acetylation of the amino group, oxidation of the pyrimidine ring nitrogens, alpha-hydroxylation of the 6-ethyl group and conjugation with glucuronic acid. The liver could be expected to be the primary site

of metabolism. The high lipophilicity of MZP should enable it to distribute widely into different body compartments, including the CNS. The several routes of metabolism should contribute to a short plasma half-life and rapid excretion. Elimination should be *via* the kidneys and in the faeces and enterohepatic recycling could be anticipated.

The aim of the present study was to assess, by experimentation *in vitro* and using animals and man, the extent to which these expectations were fulfilled. A phase I dose-escalation pharmacokinetic study would reveal any dose-dependency in the pharmacokinetics of MZP in man, enable the results of *in vitro* tests against DHFR to be related to plasma levels of MZP found *in vivo* and establish the maximum dose of MZP tolerated by man. Such a study would contribute to the rational optimization of drug dose, schedule and route of administration for a phase II trial.

2. MATERIALS AND METHODS

2.1 General

2.1.1 Solvents and reagents

Acetonitrile, dichloromethane and methanol (all HPLC grade) were purchased from BDH Chemicals Ltd. HPLC quality 1-chlorobutane was obtained from Fisons Ltd. L-cysteine, reduced glutathione and sodium dodecyl sulphate of "Biochemical" grade were obtained from BDH Chemicals Ltd, as was 1,2-dichloroethane "Spectrosol". All other solvents and reagents were of "Analar" grade and were purchased from BDH Chemicals Ltd. Ammonia solution was of SG 0.88. The water employed throughout was from an "Amsco" still. Nitrogen (zero grade) was purchased from BOC Ltd and was filtered to 0.22 microns on-line during use.

2.1.2 Reference compounds

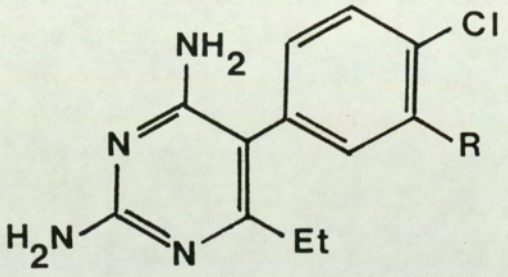
Pyrimethamine BP, etoprine and metoprine were obtained from the Wellcome Foundation Ltd. MAP, MAP-Ac, formylated MAP, m-nitropyrimethamine, pyrimethamine 2-aminopyrimidin-4-(3H)-one, MZP-N-1-oxide, MZP-N-3-oxide (Figure 2.1), MZP and MZPES were synthesized within the Pharmaceutical Sciences Institute at Aston University. Only one batch of each material was used throughout the present study.

To prepare MZP, pyrimethamine is reacted with a nitric-sulphuric acid mixture to produce m-nitropyrimethamine, which is treated with hydrazine hydrate/Raney nickel to give MAP. The amine is diazotised with sodium nitrite in hydrochloric acid then the diazonium salt is treated with sodium azide. The resultant MZP is reacted with ethanesulphonic acid to produce MZPES (176,177). The molecular weight of MZP is 289.7. One gram of MZP is equivalent to 1.38 g of MZPES.

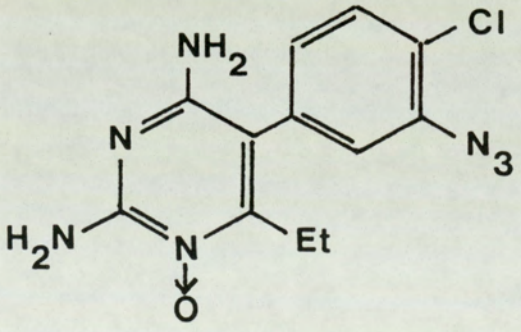
Reference compounds were stored protected from

Figure 2.1 Structures of reference compounds

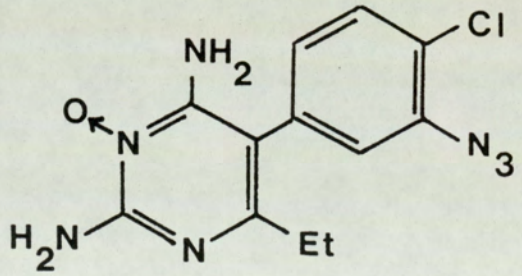
	R
MAP	NH_2
MAP-Ac	NHCOMe
FORMYLATED MAP	NHCHO
m-NITROPYRIMETHAMINE	NO_2



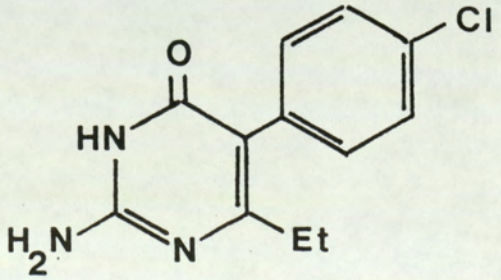
MZP-N-1-OXIDE



MZP-N-3-OXIDE



PYRIMETHAMINE 2-AMINOPYRIMIDIN-4-(3H)-ONE



light. Analysis using a variety of HPLC (Section 3.1.1) and TLC (Section 3.2) systems demonstrated that reference materials were of acceptable purity.

2.1.3 Reference Solutions

Stock 0.1% w/v methanolic solutions of the reference compounds were made with gentle heat (0.01% w/v methanolic solutions of MZP-N-1-oxide were prepared because of the poor solubility of this derivative). Aqueous solutions of MZPES were employed. All solutions were stored protected from light and were freshly prepared each month.

2.1.4 Control human urine and plasma

Twenty-four hour urine specimens were collected from a healthy volunteer who was not receiving any medication. The urine was stored at -20° in glass tubes. CPD-adenine formula 1 human plasma was obtained from the Oxford Regional Blood Transfusion Service and stored at 2 to 5° C as received in plastic bags.

2.1.5 Animals

All animals used in the course of this work were obtained from Bamtin and Kingman, Aldbrough, Horton, Yorkshire. Animals were kept in an animal house for at least a week before use to allow for acclimatization. Animals were maintained on Heygates modified rat and mouse breeding diet pellets throughout.

2.1.6 Weighings and volume measurements

All weighings were performed on an Oertling R42 4-figure balance using pre-weighed bottles. Volumes were dispensed using the appropriate "Finnpipette" (0.05 to 0.2 ml or 1.0 to 5.0 ml, Jencons Scientific Ltd), or, if 0.03 ml or less, using a 0.03 ml SGE

syringe. Pipettes were calibrated regularly by dispensing volumes of water and weighing.

2.1.7 Glassware

Volumetric flasks employed were Class A. Centrifuge tubes for extraction work were stoppered, 30 ml capacity and manufactured from borosilicate glass. Glass tubes of capacity 14 ml with tapered bases were employed to hold sample residues prior to HPLC analysis. Griffiths tubes made of glass were used in homogenize faeces and tissues.

2.1.8 pH measurements

The pH meter employed was model PW 9418 (Philips) fitted with a standard glass electrode. The instrument was calibrated before use with buffer solutions of pH 4.0, 7.0 and 9.2.

2.1.9 Ultraviolet spectrophotometric measurements

The UV spectrophotometer used was model SP 8-100 (Pye Unicam Ltd) equipped with 1 cm pathlength silica cuvettes.

2.1.10 Mixing and centrifuging

The "Whirlimixer" (Fisons Ltd) was used for vortex mixing in the analytical procedures. The Beckman "Microfuge" B, and an Heraeus instrument, were employed to centrifuge mouse and human blood samples respectively. In the analytical procedures the MSE "Minor" centrifuge was used.

2.1.11 Incubations

A Gallenkamp water-bath was employed for incubations.

2.2 Analytical

2.2.1 High pressure liquid chromatography

HPLC was the principal analytical method used in this work. Development of the HPLC methods employed is described in Section 3 and details of the specific conditions for each analysis are described later in Section 2.

The Pye Unicam LC3 system, comprising a pump and variable wavelength UV detector, was employed. Injections were made via a Rheodyne 7120 injector equipped with a 0.02 or 0.05 ml loop. The device was loaded using a Rheodyne RL syringe of capacity 0.05 ml. The analytical columns were made of stainless steel 250 mm x 4.5 mm id and were packed with C_{18} 10 micron reversed-phase material (Partisil 10 0DS or Partisil 10 ODS 2, Whatman Ltd). The columns were operated at ambient temperature. Analytical columns were protected with a stainless steel guard column 70 mm x 2 mm id containing Co: Pell ODS (Whatman Ltd). The recorder was a Philips model PM 8251/21.

2.2.2 Preparation of mobile phase

Phosphate solutions of pH 7.5, 7.0 and 5.0 were Sørensens buffers reduced in concentration to 0.02 M and prepared according to Table 2.1. Orthophosphoric acid was added to 0.02 M potassium dihydrogen orthophosphate to produce solutions of pH 4.5 and 3.0

Table 2.1 Preparation of phosphate solutions

Solution pH	7.5	7.0	5.0	4.5	3.0	
0.02 M potassium dihydrogen orthophosphate	164	392	998	1000	1000	ml
0.02 M di-sodium hydrogen orthophosphate	836	608	2	0	0	ml
Orthophosphoric acid	0	0	0	Sufficient		

To prepare mobile phase phosphate solutions were mixed with methanol volume-in-volume. When required sodium dodecyl sulphate (SDS) was incorporated as ion-pairing reagent. Eluant was degassed and filtered before use by passage under vacuum through a no 4 sintered glass filter.

2.2.3 Thin-layer chromatography

TLC separations of some 2,4-diaminopyrimidines were accomplished using plastic sheets coated with a 0.2 mm layer of silicagel (Kieselgel 60 F254), Merck no 5735). Sheets were cut into rectangles 10 cm x 20 cm and 5 mm strips of the adsorbent were removed from the longer sides. Each sheet was activated at 105°C for 1 hour before use and allowed to cool in a desiccator. Stock 0.1% methanolic solutions of each test material were prepared with gentle heating and 0.005 ml portions were applied 15 mm apart to the origin. MZP-N-1-oxide was applied as 5 x 0.01 ml of a 0.01% w/v solution, with drying between each application. Spots were dried in a stream of warm air before development.

Solvent systems were prepared v/v fresh for each analysis and allowed to equilibrate for 3 hours in standard glass tanks lined with filter paper. Each loaded sheet was placed in mobile phase to a level well below the origin, the tank lid was replaced and development was allowed to proceed in the dark to a height of 15 cm. Sheets were dried in warm air after removal from the tanks and examined under UV light at 254 and 375 nm.

2.3 Quality control samples

To prepare quality control pools, MZPES was dissolved in the minimal volume of water and incorporated into blank human plasma. For murine pharmacokinetic studies plasma was spiked with MZP at concentrations of 4.5 and 9.0 mg/L. The plasma was distributed into Eppendorf 1.5 ml microtubes and stored at -20°C. For most of the human

pharmacokinetic study the concentration of MZP in control plasma was 1.0 mg/L but for some of the earlier work the pool was diluted with blank plasma to produce samples containing MZP at 0.25 mg/L. The higher-strength quality control plasma was stored in glass tubes and the lower-strength pool was distributed into Eppendorf microtubes. Both were kept at -20°C.

2.4 Stability of MZP, MAP and MAP-Ac in plasma and urine at 37°C

Aliquots of methanolic solutions of MZP, MAP and MAP-Ac were pipetted into a glass tube and evaporated to dryness at 30°C under a stream of nitrogen. Control human plasma (10.0 ml) was added to the residue, the tube contents were vortex-mixed for 5 minutes and the spiked plasma was transferred to a corresponding tube. The nominal concentration of each material in the plasma was 9.0 mg/L. Samples were removed for analysis. Two more (stoppered) tubes were weighed, 9.2 ml of control plasma was added to each and the tubes were stopped and re-weighed. Control plasma (9.6 ml) was pipetted into a fourth tube. All tubes were stoppered, covered with silver foil and incubated at 37°C. After 5.5 and 11.0 hours tubes containing the spiked sample and the control (fourth) sample were removed from the water-bath and shaken gently to wash condensate from the walls. Plasma samples were removed, cooled and analysed. Internal standard was not added to the control sample. After 11.0 hours the tared tubes were removed from the water-bath, dried and re-weighed. The procedure was repeated using control human urine in place of the plasma. The analytical procedures employed are described in Sections 2.7 (urine) and 2.8 (plasma). The results of the experiments are presented in Section 3.6

2.5 Reaction of MZP with some cellular reducing agents

The buffers employed were based on Sørensens formulae and were prepared according to Table 2.2.

Table 2.2 Preparation of phosphate buffers

pH	7.4	5.0
0.0667 M potassium dihydrogen orthophosphate	19.6	98.8 ml
0.0667 M di-sodium hydrogen orthophosphate	80.4	1.2 ml

Buffers (2.0 ml portions) were distributed into glass tubes and 0.048 ml of aqueous MZPES solution (517.5 mg/L) was added to each tube. Aqueous solutions of reduced glutathione and cysteine (35 mg/ml) were prepared immediately before the experiments and 0.03 and 0.3 ml portions were dispensed into the buffers. Each solution was then made up to 3.0 ml with appropriate buffer. The concentration of MZP in each solution was thus 6.0 mg/L and the molar excesses (reductant/MZP) were 55 and 550 for glutathione and 1400 for cysteine. Buffer solutions containing MZP at 6.0 mg/L, but without reductant, were also prepared. Samples were removed for analysis. Two more (stoppered) tubes were weighed, 2.7 ml of water was added to each and the tubes were stoppered and re-weighed. All tubes were stoppered, covered with silver foil and incubated at 37°C. After 3.0 and 6.0 hour tubes containing MZP were removed from the water-bath and shaken gently to wash condensate from the walls. Samples were removed, cooled and analysed. After 3.0 hours the tared tubes were removed from the water-bath, dried and re-weighed. Water (0.3 ml) was removed from each and the tubes were stoppered and weighed. The tubes were returned to the water-bath and at 6.0 hours they were removed, dried and re-weighed. The analytical method was that employed for mouse plasma (Section 2.8). The results of the investigation are presented in Section 3.7.

2.6 Degradation of MZP on incubation with whole mouse liver

Whole mouse liver (100 mg portions) was weighed into a series of 15 glass tubes with tapered bases. Water (0.02 ml) was added to one tube and 0.02 ml of aqueous MZPES solution (517.5 mg/L) was added to each of the remaining tubes. The contents of each tube were mixed with a glass rod to produce homogenates containing MZP at 62.5 mg/kg. Samples (20 mg) were removed from 2 tubes, transfered to tissue grinders and immediately stored on dry-ice. The remaining tubes were covered with silver foil and incubated at 37°. After 1.5, 3.0, 4.5, 6.0, 7.5 and 9.0 hours samples (20 mg) were removed from replicate test tubes and stored as before. The control liver was sampled at 9.0 hours. Samples were analysed by the procedure described in Section 2.9. The results of the experiment are presented in Section 3.8

2.7 MZP excretion balance study in the rat

This study was designed to trace the fate of MZP following administration of the drug to male Wistar rats. Two rats were dosed at 50 mg/kg and two rats were dosed at 100 mg/kg, by the IP route. The subsequent excretion of MZP was monitored by collecting urine and faeces.

The rats were housed in glass metabolic cages for 24 hours before the start and during the course of these experiments (48 and 72 hours). Each cage contained a metal grid base such that urine and faeces fell through into pre-weighed Sterilin vials beneath. A moveable glass insert directed urine into a side-positioned vial whilst faeces fell straight through into a vial directly beneath the grid. Each cage was positioned over a bath at -5°C so that urine and faeces vials were immersed. In this way trapped samples were immediately cooled. Food and water was available to the rats *via* attached

dispensers. Control urine and faeces were collected for the 24 hours preceding the start of each experiment and pooled.

The rats were weighed before the start of each experiment and the appropriate doses of MZP were calculated. MZP (100 mg) was triturated with 5 ml of physiological saline containing 2 drops of Tween 80. The formulation was such that 0.5 ml was equivalent to a dose of 50 mg/kg for a 200 g rat. MZP (98 mg) was triturated with 4 ml of physiological saline containing 2 drops of Tween 80 to produce a formulation such that 0.5 ml was equivalent to a dose of 50 mg/kg for a rat of weight 245 g. The appropriate doses (0.5 ml or 1.0 ml) were administered with a 1 ml syringe and the rats were placed in the cages.

Samples of urine and faeces were collected at 24 (or 21), 48 and 72 hours after dosing. At each time-point urine and faeces vials were exchanged for clean ones. Each vial was re-weighed to determine the sample weight and stored at -20°C. At the end of the experiment rats were re-weighed to determine whether any change in weight had occurred.

Rat urine was analysed for MZP, MAP and MAP-Ac as follows. Each sample was thawed immediately before analysis then agitated on the vortex mixer for about 10 seconds. The sample (0.1 ml), or an aqueous dilution thereof, was made up to 2.0 ml with water. Internal standard solution (0.45 micrograms of pyrimethamine in 0.02 ml of methanol) was added followed by 0.1 ml of 10% w/v sodium hydroxide solution. Triple-distilled 1,2-dichloroethane or 1-chlorobutane:dichloromethane 96:4 (5.0 ml) was added and the tube was stoppered and shaken on the vortex mixer for 1.0 minute. The tube was centrifuged at 1900 g for 5 minutes then 3.0 ml of the organic layer was removed and evaporated to dryness at 30°C under a gentle stream of nitrogen. The tube was stored in the dark whilst awaiting examination of the contents by HPLC.

For chromatography the Partisil 10 ODS 2 column was

employed with detection at 272 nm. Methanol:phosphate (0.02 M, pH 3.0) 79:21, containing 0.02 M SDS, was used as mobile phase at a flowrate of 1.4 ml/min. Immediately before HPLC 0.1 ml of mobile phase was added to the residue in the tube and the tube contents were vortex-mixed for 1.0 minute. The injection volume was 0.02 ml.

Calibration samples were prepared by pipetting aliquots of methanolic dilutions of the stock MAP and MAP-Ac solutions into extraction tubes and evaporating at 30°C under nitrogen. MZP was incorporated as aliquots of dilutions of the stock aqueous MZPES solution, the concentration sequence being as for MAP and MAP-Ac. Control urine (0.1 ml) was added to each tube and to a further tube not containing test materials. The contents of each tube were extracted and chromatographed. The quantity of internal standard added in the analytical procedure corresponded to the mid-point of the calibration sequence (4.5 mg/L). Curves relating peak height ratios (test materials/internal standard) to test material concentrations (mg/L of urine) were prepared by linear regression analysis. Standard curves were linear to at least 9 mg/L.

Faeces were analysed as follows. The sample was homogenized in a mortar whilst still frozen then about 75 mg of thawed wet material was accurately weighed into a Griffiths tube. Saline (0.9% w/v, 1.0 ml) was added, the sample was ground and the suspension was transferred to an extraction tube. The grinder was rinsed with another 1.0 ml of saline and the rinsing was added to the contents of the extraction tube. The subsequent procedure was as for urine; triple-distilled dichloromethane was used for extraction. The procedure was modified to allow measurement of MZP at higher concentrations. Faeces (30 mg) was suspended in 10 ml of saline and 1.0 ml of the suspension was taken through the analytical procedure. Calibration samples for MZP were prepared by spotting aqueous MZPES solution into control rat faeces. The method described for rat urine was used to calibrate the procedure for MAP and MAP-Ac.

The results of the excretion balance study are presented in Section 3.9.

2.8 MZP murine plasma pharmacokinetics

Murine plasma pharmacokinetic studies were conducted using non-tumour-bearing male Balb C mice. The animals were dosed with MZP at 10 and 20 mg/kg by the IP route. An assessment of oral bioavailability, relative to IP injection, was made at the lower dose.

Mice were weighed on the evening before each study and the appropriate doses of MZP were calculated. For the IP studies 345 mg of MZPES was dissolved in 50 ml of water with warming, the solution was cooled then passed through a sterile 0.45 micron filter into a sterile Universal bottle. This dose formulation was such that 0.1 ml of the solution was equivalent to a dose of 20 mg/kg for a 25 g mouse. MZPES (86.3 mg) was dissolved in 25 ml of water and the solution was sterilized as before. 0.1 ml of this formulation was equivalent to a dose of 10 mg/kg for a 25 g mouse. The appropriate volumes, adjusted according to the weight of each mouse, were administered using a hypodermic syringe. For the oral bioavailabity study the formulation employed was as for the IP study at 10 mg/kg. Oral dosing, again normalized for the weight of each mouse, was accomplished by sliding an oral dispensing tube into the stomach of each mouse and ejecting the dose using a syringe. In all experiments the numbers of mice dosed were such that 5 animals could be evaluated at each time-point.

Mice were housed in standard size cages (5 mice per cage) with adequate supplies of food and water during the experiments. Blood collection tubes (Eppendorf 1.5 ml microtubes) containing 0.1 ml of 3% tri-sodium citrate as anticoagulant were cooled on ice before use.

Mice were sampled 0.25, 0.50, 0.75, 1.50, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 12.00 and 24.00 hours after

administration of MZP (total 65 mice). At sampling times each mouse was anaesthetized with a Boyles apparatus using halothane and nitrous oxide as the anaesthetic and 0.9 ml of blood was removed by cardiac puncture from the right ventricle. The time at which the blood was in the syringe was recorded. The sample was transferred to a collection tube and mixed with the anticoagulant before centrifuging for 2 minutes. The supernatant plasma was removed to a corresponding tube and stored at -20°C whilst awaiting analysis. Control plasma samples were obtained from untreated mice. Mice were killed by dislocation of the neck whilst still under anaesthesia.

Each plasma sample was thawed immediately before analysis then agitated on the vortex mixer for about 10 seconds. The sample (0.1 ml) was diluted to 0.5 ml with water. Internal standard solution (0.45 micrograms of pyrimethamine in 0.02 ml of methanol) was added followed by 0.1 ml of 10% wv sodium hydroxide solution. 1-chlorobutane:dichloromethane 96:4 or triple-distilled dichloroethane (5 ml) was added and the tube was stoppered and shaken on the vortex mixer for 1 minute. The tube was centrifuged at 1900 g for 5 minutes then 3.0 ml of the organic layer was removed and evaporated to dryness at 30°C under a gentle stream of nitrogen. The tube was stored in the dark whilst awaiting examination of the contents by HPLC.

Chromatographic conditions were used as for rat urine (Section 2.7) but in order to minimize retention times methanol:phosphate (0.02 M, pH 3.0) 83:17, containing 0.02 M SDS, was employed as mobile phase. Calibration curves for MZP, MAP and MAP-Ac were prepared by the method described for rat urine.

The results of the mouse plasma pharmacokinetic study are presented in Section 3.10. Concentration-time data were transformed into pharmacokinetic parameters using a Lotus Symphony spreadsheet developed by Dr. J.A. Slack. AUC values were estimated by the trapezoidal method until the end of the sampling period (AUC_{trap}) and by

extrapolation of time from zero to infinity (AUC_{0-inf}). The oral bioavailability, or amount of the administered dose which reaches the systemic circulation (F), was obtained from

$$F = \frac{AUC_{po\ route}}{AUC_{ip\ route}} \qquad (AUC = AUC_{trap})$$

By convention the denominator should be the AUC obtained when the IV (bolus) route of administration is used. In studies using mice administering a drug by the IV route is technically difficult therefore the AUC following an IP administration of MZP was substituted into the bioavailability calculation.

2.9 MZP murine tissue pharmacokinetics

The disposition of MZP in some tissues of the mouse was studied using non-tumour bearing male Balb C mice. The animals were dosed at 20 mg/kg (free base) by the IP route and levels of MZP and MAP were measured in liver, brain, heart, lung and kidney over 48 hours.

Mice were prepared and housed as in the mouse plasma study (Section 2.8). For dosing 86.3 mg of MZPES was dissolved in 25 ml of water and the solution was sterilized by filtration. 0.2 ml of this formulation was equivalent to a dose of 20 mg/kg for a 25 g mouse. Dosing was normalized for the weight of each mouse and accomplished using a hypodermic syringe. The number of mice dosed was such that 2 animals could be sampled at each time-point.

Mice were sampled 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0 and 48.0 hours after dosing (total 20 mice). At sampling times mice were killed by dislocation of the neck and the time-point was recorded. The organs were immediately removed and transferred to pre-weighed Sterilin vials. Blood was squeezed out of each heart before storing this organ. The vials were re-weighed in order

to determine the sample weights and stored at -20°C whilst awaiting analysis. Control organ samples were dissected from untreated mice by the same procedure.

Frozen lung was cut into small pieces with scissors and fat was removed from frozen kidney. The sample was homogenized in a mortar whilst still frozen then about 20 mg of thawed wet material was accurately weighed into a Griffiths tube. Saline (0.9% w/v, 0.5 ml) was added followed by internal standard solution (0.45 micrograms of pyrimethamine in 0.02 ml of methanol). The sample was ground and the suspension was transferred to an extraction tube. The Griffiths tube was rinsed with another 0.5 ml of saline and the rinsing was added to the contents of the extraction tube. The subsequent analytical procedure was as for mouse plasma (Section 2.8); 1-chlorobutane:dichloromethane 96:4 was employed as extraction solvent. Calibration samples for the azide were prepared by spotting aqueous MZPES solution into control mouse liver. Calibration curves were linear to at least 45 mg of MZP per kg of liver. Curves were prepared for MAP and MAP-Ac by the method described for rat urine, with introduction of the standard materials to the Griffiths tubes.

The results of the disposition study are presented in Section 3.10. The Lotus Symphony spreadsheet was used to calculate the apparent half-lives for elimination of MZP from tissues.

2.10 Clinical pharmacokinetics of MZP

A human pharmacokinetic study formed part of the phase I clinical trial of MZP. The trial was a collaborative exercise conducted in Birmingham and at Charing Cross Hospital in London. Pharmacokinetic studies on patients treated in the Birmingham arm of the trial were performed as part of the present work. Results from the Charing Cross arm are included in Section 3.11 to enable conclusions

to be reached concerning the overall results of the clinical pharmacokinetic study. The eligibility of patients for entry into the trial was based on standard guidelines (178). All patients had histologically-confirmed progressive cancer refractory to all known forms of therapy. Requirements were imposed for adequate bone marrow, hepatic and renal functions as determined by standard tests.

MZPES was formulated for the dose escalation study as a 1% w/v solution in water for injections sealed in 5 ml glass ampoules and sterilized by filtration (81). Ampoules were stored in a refrigerator at 2 to 5°C and were protected from light. Immediately before use the appropriate volume of the solution was injected into 500 ml of 5% dextrose injection BP (Steriflex, Boots) and mixed. If the volume added was more than 35 ml the identical volume of dextrose injection was removed prior to addition. The infusion was protected from light. It is stable for at least 24 hours in the dark at room temperature (179). The infusion was administered over approximately 1 hour and the exact infusion time was recorded.

The starting dose of 5.4 mg/m² was chosen as it represented approximately one-tenth of the mouse equivalent IV LD_{10} (18 mg/kg, Table 1.3). Dose escalations proceeded as a modified Fibonacci sequence through 11, 18, 27, 38, 50, 67, 83, 105, 125, 150, 177, 200, 210, 215, 250, 300, 360, 400 and 460 mg/m². A minimum of 4 weeks was allowed between successive courses of treatment to individual patients.

In the Birmingham arm of the trial samples were taken by Dr. J.A. Slack and Dr. S.K. Wong. Blood was sampled just before the infusion, immediately after the infusion and at 1.33, 1.67, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 12.00, 24.00 and 48.00 hours after the start of the infusion. Samples were taken from an indwelling Venflon with an attached 3-way tap. The times were recorded when blood was in the syringe. Clotting of blood in the line between sampling times was prevented by injection of 0.5 ml

of heparin (1000 units/ml) after each sampling. At sampling times 2 ml of blood was taken off to waste then a 10 ml sample was drawn into a plastic syringe. The blood was immediately transferred to a heparinized blood sample tube (Sterilin Ltd), mixed and placed on ice. The sample was centrifuged at 2000 rpm at 3°C for 10 minutes. The supernatant plasma was pipetted into Sterilin sample tubes and stored at -20°C whilst awaiting analysis for MZP concentration. Each sample was thawed immediately before analysis then agitated on the vortex mixer for about 10 seconds. The sample (0.5 ml) was diluted to 0.8 ml with water. Internal standard solution (0.02 ml of methanol containing an amount of pyrimethamine corresponding to the mid-point of the calibration sequence) was added followed by 0.1 ml of 10% w/v sodium hydroxide solution. 1-chlorobutane:dichloromethane 96:4 (5.0 ml) was added and the tube was stoppered and shaken on the vortex mixer for 1 minute. The tube was centrifuged at 1900 g for 5 minutes then 4.5 ml of the organic layer was removed and evaporated to dryness at 30°C under a gentle stream of nitrogen. The tube was stored in the dark whilst awaiting examination of the contents by HPLC.

For chromatography the Partisil 10 ODS 2 column was employed with detection at 272 nm. Methanol:phosphate (0.02 M, pH 3.0) 83:17, containing 0.02 M SDS, was used as mobile phase for most of the human pharmacokinetic work but the volume ratio was altered to 81:19 or 79:21 for some of the later work when some deterioration in column performance was encountered. The flowrate was 1.4 ml/min. Immediately before HPLC 0.25 ml of mobile phase was added to the residue in the tube and the tube contents were vortex-mixed for 1.0 minute. The injection volume was 0.05 ml.

For preparation of calibration samples 0.5 ml of blank plasma was pipetted into a tube, then 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 ml aliquots of MZPES solution was added to further 0.5 ml blank plasma samples. Each mixture, except the last, was made up to 0.8 ml with water

and 0.02 ml of internal standard solution was added to each tube. Calibration curves covered the ranges 0 - 0.48 mg/L to 0 - 34.56 mg/L of MZP in plasma. The quantity of internal standard added corresponded to the mid-point of each calibration sequence. The contents of each tube were extracted and chromatographed by the standard procedure. Curves relating peak height ratios (MZP/pyrimethamine) to MZP concentrations (mg/L of plasma) were prepared by linear regression analysis.

The Lotus Symphony spreadsheet was used to generate plots of MZP plasma concentration (arithmetic and ln) versus time. NONLIN (182) was employed to calculate AUC_{trap} AUC_{0-inf} (mgL/hr), central compartment volume (L), apparent V_d at steady-state (post distribution) (L), distribution and terminal elimination half-life (hr) values and the most appropriate model to which the data could be fitted in each study. Plasma clearance (L/hr) was calculated from dose/AUC_{trap}.

2.11 General analytical protocol

The analysis of each series of samples was preceded by a pilot measurement on the sample expected to contain the highest level of drug. A calibration curve embracing all encountered levels of the analyte could then be constructed. Within each test series the same lot of mobile phase was used for reconstitution of sample residues and column elution. The sequence of operations was: preparation of first calibration curve; analysis of first set of duplicates; analysis of second set of duplicates; preparation of second calibration curve.

Blank samples, taken before dosing with MZP, were included in each test series in order to detect possible interfering endogenous substances or co-administered drugs; internal standard was not added to these samples. An "external" standard solution, containing MZP, MAP, MAP-Ac

and pyrimethamine in mobile phase, was injected onto the column at intervals in order to locate analyte peaks with certainty. The height of each test peak and each pyrimethamine peak was measured from a tangent drawn across the troughs immediately on either side of the peak. Quantification was achieved from the peak height ratio of test material to internal standard and reference to the standard curve. Reagent blank analyses, using water in place of sample, were run whenever a batch of alkali, extraction solvent or mobile phase was changed. The only interference from these sources was that due to impurities in dichloroethane; this is discussed in Section 3.1.1.

Within each test series the sample found to contain the highest level of MZP was re-analysed without internal standard in order to determine whether a metabolite of MZP was eluted at the retention time of pyrimethamine. In no such sample was this found to be the case. The assumption that any such metabolite would accompany the highest level of MZP may be unwarranted, but internal standard peak heights remained consistent within each test series thus interference by a metabolite eluting with pyrimethamine could be discounted.

3 RESULTS AND DISCUSSION

3.1 Analysis of MZP, MAP and MAP-Ac in animal fluids and tissues

3.1.1 Assay development

In order to allow a study of the pharmacokinetics of MZP a specific HPLC method was developed for the quantitative analysis of the drug. MAP and MAP-Ac were putative metabolites of MZP thus initially an attempt was made to separate MZP from these two compounds. The chromatographic behaviour of pyrimethamine, metoprine, etoprine, formylated m-aminopyrimethamine, m-nitropyrimethamine, MZP-N-1-oxide, MZP-N-3-oxide and the 2-aminopyrimidin-4 (3H)-one derivative of pyrimethamine was studied concurrently.

Levin et al (153) measured metoprine in plasma and tissues using a reversed-phase column with isocratic methanol:phosphate (0.02M, pH 7.5) 65:35 as mobile phase and this system was used as the basis for development of a separation in the present work. Table 3.1 lists the wavelengths of some principal UV peaks and corresponding specific absorbance (A 1%, 1 cm) values for MZP and some congeners in methanol:phosphate (0.02M, pH 7.5) 65:35. For mobile phase at near-neutrality 288 nm was considered to be a suitable compromise detection wavelength. Table 3.2 presents column capacity factors (k^1) and retention times (rt) using ratios of methanol:phosphate (0.02 M pH 7.5) from 55:45 to 70:30 at a flowrate of 1.4 ml/min on a Partisil 10 ODS column. The aim was to achieve a retention time for MZP of between 6 and 10 minutes and Table 3.2 shows that methanol:phosphate (0.02M, pH 7.5) 60:40 fulfilled this objective and separated the drug from MAP and MAP-Ac. Pyrimethamine was identified as a possible internal standard. A mobile phase flowrate of 1.4 ml/min was used throughout the present work.

Although the aforementioned system produced satisfactory separation of MZP, MAP, MAP-Ac and pyrimethamine it was considered desirable to take advantage of the

Table 3.1 Ultraviolet spectral data for MZP and some congeners in methanol:phosphate (0.02M, pH 7.5) 65:35

Material	Principal peak wavelength (nm)	A(1%, 1 cm)
MZP	287	406
MAP	290	449
MAP-Ac	287.5	342
Pyrimethamine	287	404
Etoprine	287	354
m-Nitropyrimethamine	285	381
Formylated m-aminopyrimethamine	287	310
Pyrimethamine 2-aminopyrimidin -4(3H)-one	280	310

inherent high selectivity of the ion-pair partition process (180). Ion-pairs can be formed between a solute ion and an oppositely-charged ion. In the case of a base the partition equilibrium can be represented by

$$B^+ \text{ aq} + P^- \text{ aq} \rightleftharpoons (B^+ P^-) \text{ org}$$

where B^+ is the protonated form of the base and P^- is the anion of an acid that is to be used as the pairing reagent. In chromatography a reversed-phase bonded material can be used to sorb ion-pairs from an aqueous eluant (180). In a preliminary investigation eluants of composition methanol:phosphate (0.02M) 65:35, with the phosphate at pH values of 7.5, 4.5 and 3.0, were prepared, each with and without 0.01 M SDS. Comparison of capacity factors for test materials in the absence and presence of 0.01 M SDS suggested that when using pH 7.5 solution ion-pair formation did not occur. When pH 4.5 solution was used the production of ion-pairs was uncertain. At pH 3.0 peaks for MZP and the related compounds were split in the presence of 0.01 M SDS, thus ion-pair formation was perhaps partial. The proportion of a base that is protonated depends upon its pK value as well as upon the

Table 3.2 Capacity factors and retention times for MZP and some congeners using various ratios of methanol:phosphate (0.02 M, pH 7.5) as mobile phase

Ratio (methanol:phosphate)	55:45		60:40		62.5:37.5		70:30	
Material	k^1	rt	k^1	rt	k^1	rt	k^1	rt
MAP-Ac	1.81	4.50	1.33	3.96	1.26	3.77	0.86	2.97
MAP	2.87	6.17	2.14	5.02	1.67	4.53	1.19	3.51
Pyrimethamine	4.81	9.30	3.07	6.93	2.61	6.13	1.59	4.15
MZP	6.88	12.67	4.37	8.93	3.79	7.97	2.05	4.88
m-Nitropyrimethamine	3.50	7.20	2.41	5.65	2.01	5.00	1.33	3.73
Formylated m-aminopyrimethamine	1.75	4.50	1.43	3.99	1.20	3.73	0.90	3.05
Pyrimethamine 2-aminopyrimidin-4 -(3H)-one	1.62 2.38	4.20 5.40	1.45 1.89	3.93 4.63	1.12 1.47	3.53 4.10	0.99 1.12	3.18 3.39

Column Partisil 10 ODS , flowrate 1.4 ml/min

rt - minutes

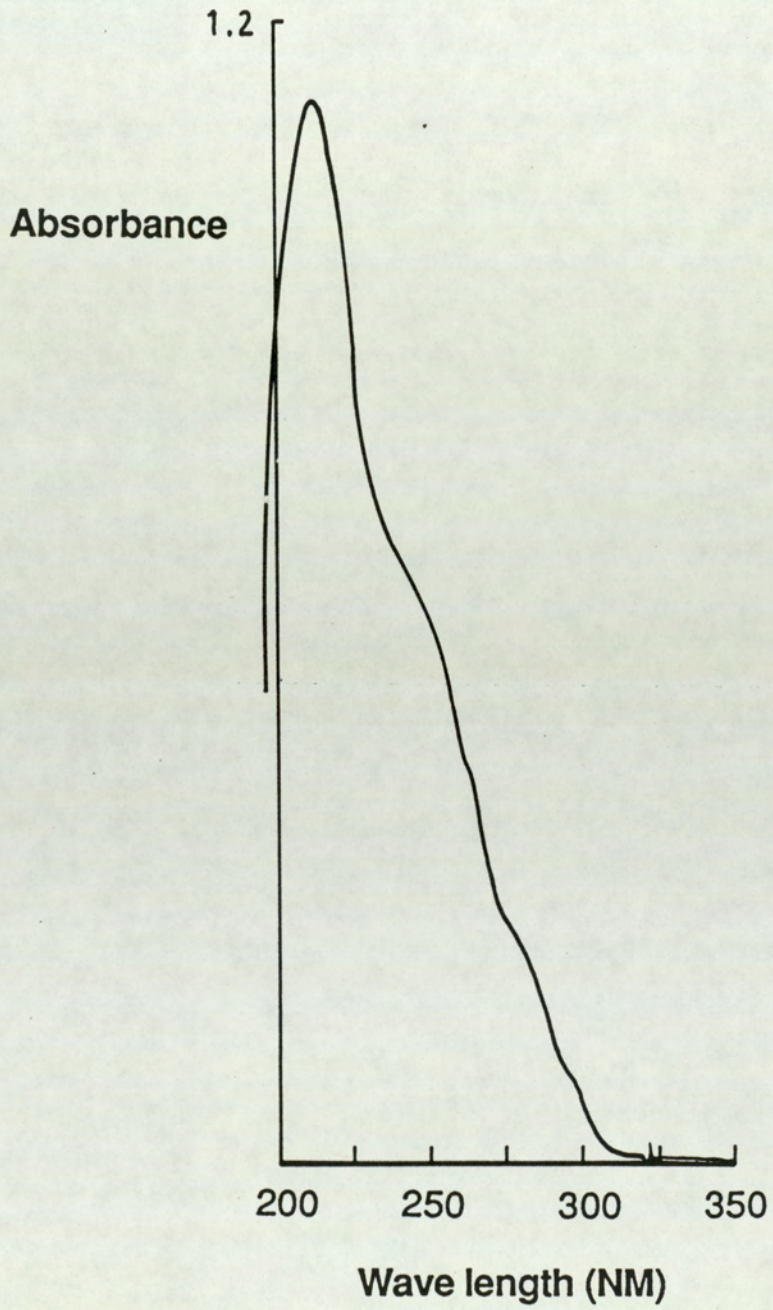
All figures are the mean of 3 determinations

pH of the solvent, but protonation, thus ion-pair formation in the presence of excess of a counterion, should be virtually complete under strongly acidic conditions. The pK_a values of the lipophilic 2,4-diaminopyrimidines are close to 7 (Table 1.1), thus these compounds will exist in both the ionized and unionized forms at near-neutral pH. The use of an acidic eluant was therefore favoured in order to minimize the possibility of concurrent chromatography of ionized and neutral species.

For subsequent work a Partisil 10 0DS 2 column was employed and the concentration of SDS in mobile phase was increased to 0.02 M. Table 3.3 presents chromatographic data for MZP and some related compounds using methanol:phosphate (0.02 M, pH 3.0) 79:21, with and without 0.02 M SDS, as mobile phase. The quotients k^1_{SDS}/k^1 are generally within the range 3.4 to 5.9 and clearly demonstrate the formation of ion-pairs. Variation of the proportion of methanol to phosphate (0.02 M, pH 3.0), with 0.02 M SDS present, was investigated and Table 3.4 shows that ratios within the range 79:21 to 83:17 were suitable for the analysis of MZP, MAP, MAP-Ac and pyrimethamine. Ion-pair partition chromatography, using 0.02 M SDS as counterion, was adopted for the greater part of this study. A ratio of 75:25 was required to separate MZP, MZP-N-1-oxide and MZP-N-3-oxide but this eluant did not achieve satisfactory resolution of the N-oxides from MAP, MAP-Ac and pyrimethamine. The retention time of MZP was rather long (15 min) using mobile phase of this composition and the effect of increasing the aqueous component above 25% was not investigated.

Table 3.5 lists the wavelength of some UV peaks and the corresponding A(1%, 1 cm) values for MZP and some congeners in acidic mobile phase containing 0.02 M SDS. Although MZP showed a peak only at 212 nm (Figure 3.1) the chromophore was strong at 272 nm and this was chosen as the detection wavelength for MZP, MAP and MAP-Ac.

Figure 3.1 Ultraviolet spectrum of MZP in methanol: phosphate (0.02M,pH 3.0) 79:21, containing 0.02M SDS



Concentration 0.00075%, cell pathlength 1cm

Table 3.3 Capacity factors and retention times for MZP and some congeners using methanol: phosphate (0.02 M, pH 3.0) 79:21, with and without 0.02 M SDS, as mobile phase

Material	k^1_{SDS}	rt_{SDS}	k^1	rt	$\frac{k^1_{SDS}}{k^1}$
MAP-Ac	1.32	4.13	0.37	2.35	3.57
MAP	1.90	5.17	0.52	2.61	3.65
Pyrimethamine	2.70	6.59	0.79	3.08	3.42
MZP	3.95	8.82	1.11	3.63	3.56
Etoprine	3.99	8.88	0.96	3.37	4.16
Metoprine	2.75	7.50	0.57	2.75	4.82
m-Nitropyrimethamine	2.12	5.62	0.49	2.71	4.33
Formylated m-amino-pyrimethamine	1.24	4.03	0.21	2.21	5.90
Pyrimethamine 2-amino-pyrimidine-4(3H)-one	1.03 1.17	3.66 3.91	0.58 0.94	2.87 3.53	1.78 1.25

Column Partisil 10 ODS 2, flowrate 1.4 ml/min

rt - minutes

All figures are the mean of 3 determinations

Absorbance of the N-oxides at this wavelength was weak in comparison with the other compounds examined. The absorbance of the mobile phase at 272 nm did not differ appreciably from that of water at this wavelength, thus no limitation on sensitivity of measurement was imposed by UV opacity of the mobile phase.

In all chromatographic systems studied (Tables 3.2, 3.3 and 3.4) MAP-Ac and formylated MAP showed least affinity for the columns. Capacity factors for these compounds were similar in each system and overall they lay within the range 0.2 to 2.1. MAP and m-nitropyrimethamine showed higher affinity for the hydrophobic bonded phases and these two compounds exhibited similar retention behaviour. The chromatography of MAP-Ac and formylated MAP relative to that of MAP was not as anticipated. Acylation of an amino group normally reduces the

Table 3.4 Capacity factors and retention times for MZP and some congeners using various ratios of methanol:phosphate (0.02 M, pH 3.0), containing 0.02 M SDS, as mobile phase

Ratio (methanol:phosphate)	75:25		76:24		79:21		83:17		90:10	
Material	k^1	rt	k^1	rt	k^1	rt	k^1	rt	k^1	rt
MAP-Ac	2.02	5.75	1.69	5.17	1.32	4.13	0.86	3.35	0.39	2.45
MAP	3.08	7.75	2.58	6.87	1.90	5.17	1.17	3.91	0.51	2.65
Pyrimethamine	4.53	10.50	3.78	9.17	2.70	6.59	1.58	4.65	0.69	2.97
MZP	7.03	15.25	5.76	12.98	3.95	8.81	2.21	5.77	0.88	3.31
MZP-N-1-oxide	2.42	6.50	-	-	-	-	-	-	-	-
MZP-N-3-oxide	3.74	9.00	-	-	-	-	-	-	-	-

Column Partisil 10 ODS 2, flowrate 1.4 ml/min

rt - minutes

All figures are the mean of 3 determinations

Table 3.5 Ultraviolet spectral data for MZP and some congeners in acidic mobile phase

Material	Peak wavelength (nm)	A(1%, 1 cm)
MZP	212	1507
MAP	276	317
MAP-Ac	270 (inflexion)	275
Pyrimethamine	273 (inflexion)	315
Etoprine	270 (inflexion)	301
Metoprine	270 (inflexion)	305
m-Nitropyrimethamine	212	1387
Formylated m-amino-pyrimethamine	210	1600
Pyrimethamine-2-amino-pyrimidin-4(3H)-one	283	345
MZP-N-1-oxide	247	1097
MZP-N-3-oxide	227	1180

The solvent for the N-oxides was methanol:phosphate (0.02 M, pH 3.0) 83:17 containing 0.2 M SDS. For the other materials the solvent was altered inasmuch as the ratio of methanol to phosphate was 79:21.

polarity of the parent molecule, thus the derivatives of MAP would be expected to reside on a reversed-phase column longer than the free amine, but the opposite effect was observed. Pyrimethamine, then MZP, emerged after m-nitropyrimethamine.

The solvent system used most extensively in the present work was methanol:phosphate (0.02 M, pH 3.0) 83:17 containing 0.02 M SDS. Capacity factors on the Partisil 10 ODS 2 column eluted with this solvent were 1.58 and 2.21 for pyrimethamine and MZP respectively (Table 3.4). All the systems demonstrated that MAP and MAP-Ac are more polar than MZP. The amine and its acetylated derivative were putative metabolites of MZP

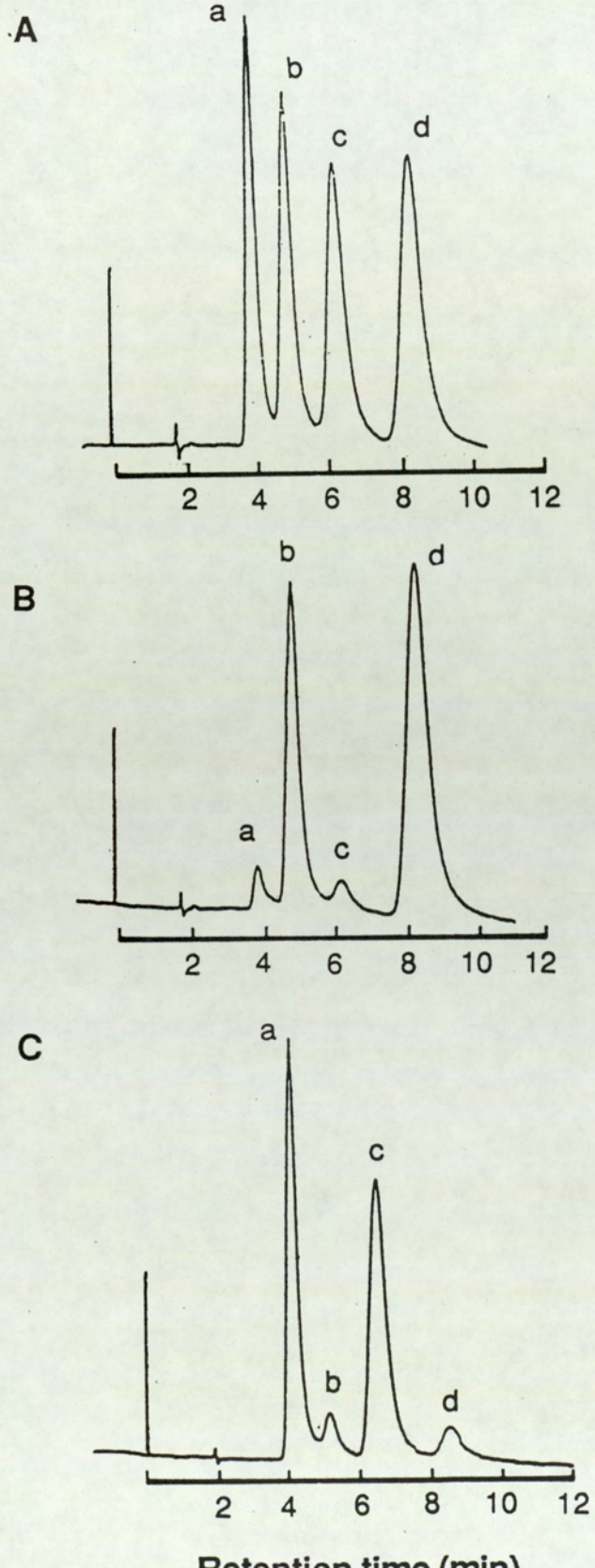
(Section 1.10.4) and it is a common observation (170) that metabolites are more polar than parent drugs. The capacity factors of etoprine were close to those of MZP (Table 3.3). The system described in Tables 3.2 and 3.3 appeared to separate the isomeric aminopyrimidones produced upon acid hydrolysis of pyrimethamine (181).

Figure 3.2 shows a chromatogram of equal quantities of MAP-Ac, MAP, pyrimethamine and MZP using methanol: phosphate (0.02 M, pH 3.0) 79:21, containing 0.02 M SDS, as mobile phase. This figure also shows separation under the same conditions with each component in the presence of tenfold excesses of the materials that emerge as neighbouring peaks. Peaks were well-resolved and the sensitivity of detection of each in the presence of excesses of the others was acceptable.

In order to ensure that conditions optimal for the separation of MAP-Ac, MAP, pyrimethamine and MZP had been achieved the effect of alteration of the pH of the aqueous component of the mobile phase was investigated. Solvents of composition methanol:phosphate (0.02 M) 79:21, with and without 0.02 M SDS, and with the phosphate component at pH 7.0 and 5.0, were prepared. Capacity factors and retention times are presented in Table 3.6. At pH 7.0 each chromatogram showed a major peak and several subsidiary peaks. Peaks were not reproducible upon repeat injection and ion-pair formation was not clearly evident. At pH 5.0, without SDS, spurious peaks subsidiary to the main peaks were again seen, but ion-pairs were apparently produced with SDS. There was no clear advantage in using phosphate pH 5.0 however thus various ratios of methanol: phosphate (0.02 M, pH 3.0), containing 0.02 M SDS, were used for all subsequent work in the present study.

In order to separate MZP from plasma or urine and render it amenable to analysis by HPLC a protein-precipitation procedure was firstly evaluated. Levin et al (153) precipitated endogenous materials with a methanol:phosphate (pH 7.5) mixture when measuring metoprine in plasma and

Figure 3.2 Chromatograms of MAP-Ac, MAP, pyrimethamine and MZP



Column Partisil 10 ODS 2, mobile phase methanol:phosphate (0.02M, pH 3.0) 79:21, containing 0.02M SDS, flowrate 1.4 ml/min, detection wavelength 272nm

		Column loading (ng)		
	Peak	A	B	C
MAP-Ac	a	800	80	800
MAP	b	800	800	80
Pyrimethamine	c	800	80	800
MZP	d	800	800	80

Table 3.6 Capacity factors and retention times for MZP and some congeners using methanol:phosphate (0.02 M, pH 7.0 and 5.0) 79:21, with and without 0.02 M SDS, as mobile phase.

Phosphate pH	7.0					5.0				
Material	k'_{SDS}	rt_{SDS}	k'	rt	$\frac{k'_{SDS}}{k'}$	k'_{SDS}	rt_{SDS}	k'	rt	$\frac{k'_{SDS}}{k'}$
MAP-Ac	1.22	4.00	1.22	4.00	1.00	0.94	3.50	0.56	2.80	1.68
MAP	1.50	4.50	1.50	4.50	1.00	1.80	5.00	0.94	3.50	1.92
Pyrimethamine	1.67	4.80	1.67	4.80	1.00	2.80	6.80	1.78	5.00	1.57
MZP	1.78	5.00	1.78	5.00	1.00	4.00	9.00	1.78	5.00	2.25

Column Partisil 10 ODS 2, flowrate 1.4 ml/min

rt - minutes

All figures are the mean of 3 determinations

brain. In the present work control plasma was spiked with MZP and pyrimethamine, methanol:phosphate (0.02 M, pH 3.0) 79:21, containing 0.02 M SDS, was mixed with the plasma and the mixture was centrifuged. The procedure was repeated using unspiked plasma and the supernatant liquids from both samples were examined by HPLC. Although the control chromatogram showed no peaks that interfered with MZP or pyrimethamine the sensitivity of measurement was poor (about 600 mg/L) because it was not possible to introduce a concentration step into the method. Potential co-precipitation of materials of interest was envisaged, thus the procedure was abandoned in favour of organic solvent extraction.

Aliquots of methanolic dilutions of the stock solutions of MZP, pyrimethamine, MAP and MAP-Ac were evaporated to dryness together in a tube on a boiling water-bath and control urine was added. Sodium hydroxide solution (pH of sample about 13), then 1,2-dichloroethane were added and the tube contents were vortex-mixed and centrifuged. An aliquot of the organic (lower) layer was removed and evaporated to dryness on a boiling water-bath. The residue was reconstituted in methanol:phosphate (0.02 M, pH 7.5) 60:40 and examined by HPLC. The procedure was repeated using unspiked urine. All the added test materials could be detected in the test extract but some interference, apparently from co-extracted endogenous materials, was evident by examination of the chromatogram of the control extract. Recovery of test materials was very low, about 30% for MZP. Recovery remained low when acetonitrile was substituted for mobile phase as reconstitution solvent and when dimethyl formamide was used instead of dichloroethane as extraction solvent. When ethyl acetate was employed as extraction solvent excessive quantities of endogenous materials were extracted with test materials.

The original procedure was repeated with the standard solutions and the dichloroethane extract evaporated to

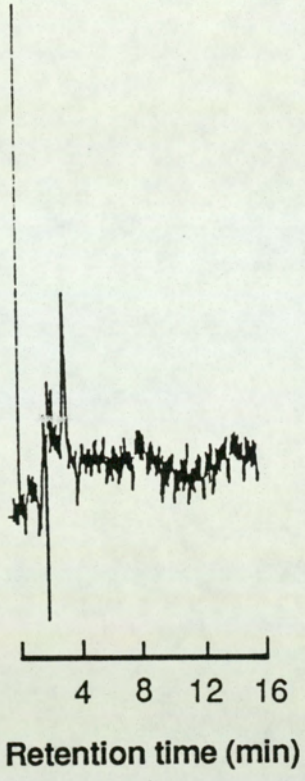
dryness at 30% under nitrogen. Recovery of added standard materials was greatly improved. At high temperatures the materials appeared to adhere to glass surfaces and were rendered poorly soluble. No further improvement in recovery was achieved by extracting twice with dichloroethane, or by extending beyond 1 minute the mixing time of dichloroethane with sample and residue with mobile phase.

Upon further consideration of the intended applications of the proposed procedure it was decided that the interference would set an unacceptable limit on the sensitivity of measurement of MZP. The reagents were examined and dichloroethane was found to be the source of the unwanted peaks. The solvent was improved by triple-distillation from glass but the quality of the distillate was erratic and the distillation procedure was tedious to perform.

During the course of this work Timm and Weidekamm (149) reported on the use of 1-chlorobutane:dichloromethane 96:4 v/v to extract pyrimethamine from plasma, thus this mixture was evaluated for use for MZP. The recovery of MZP was excellent (97%, Table 3.7). The solvent forms the upper layer when mixed with an aqueous phase, thus can be removed with ease with no contamination from urine or plasma, and the solvent evaporates faster than dichloroethane. The solvent did tend to form emulsions with plasma however, but this problem could be eliminated by increasing the ratio of solvent to sample.

Chromatograms of control rat urine and of the urine of an MZP-treated rat (both obtained by the standard procedure, Section 2.7) are shown in Figure 3.3. The former trace is essentially free from peaks due to endogenous compounds at the retention times of MAP, pyrimethamine and MZP. All endogenous peaks appeared in the first three minutes after injection. A reagent blank run under identical conditions contributed only to the short retention time peaks.

Figure 3.3 Chromatograms of rat urine extracts



1-Chlorobutane: dichloromethane 96:4 was used as extraction solvent

A-Control urine extract (no added internal standard).

B-Urine extract from rat dosed with MZP (d), with internal standard (pyrimethamine) added (c). Other peaks correspond to MAP-Ac (a) and MAP (b).

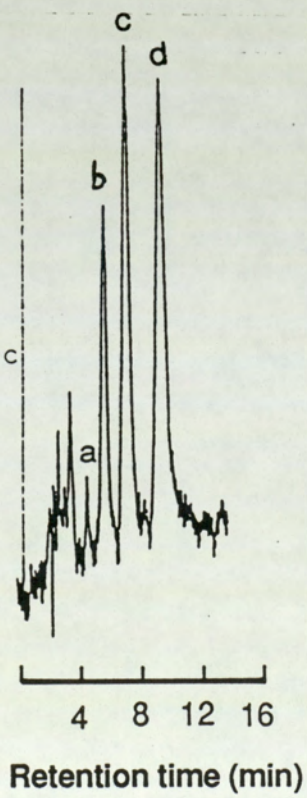


Table 3.7 Recovery of MZP, MAP, MAP-Ac and internal standard (concentrations 4.5 mg/L) from urine, using 1,2-dichloroethane and 1-chlorobutane: dichloromethane 96:4 as extraction solvents

Material	MZP	MAP	MAP-Ac	Internal standard (pyrimethamine)
Extraction solvent 1,2-dichloroethane				
Mean recovery (%)	91.3	93.8	-	77.0
CV (%)	4.5	4.8	-	4.6
n	6	6	-	6
Extraction solvent 1-chlorobutane:dichloromethane 96:4				
Mean recovery (%)	97.4	95.2	48.4	100.3
CV (%)	3.8	4.4	6.2	2.9
n	8	6	8	8

To determine the recovery of MZP, MAP and pyrimethamine aliquots of methanolic dilutions of the stock MZP and MAP solutions, containing 0.45 micrograms of each material, were evaporated to dryness together in extraction tubes at 30°C under a stream of nitrogen. Fresh control human urine (0.1 ml) was added to each tube. The contents of each tube were extracted with dichloroethane and chromatographed by the standard procedure. The recovery of analytes and internal standard was calculated by comparison of test peak heights with the heights of peaks produced by an "external" standard solution containing 54 ng of MZP, MAP and pyrimethamine in 0.02 ml of mobile phase, the theoretical concentration of each component if extraction was complete. The results, in Table 3.7, show that the extraction efficiency for pyrimethamine was 77% and for MZP and MAP exceeded 90%. MZPES had become available when the recovery was determined using 1-chlorobutane:dichloromethane 96:4 as extraction solvent thus the azide was incorporated into control urine as the salt. A stock aqueous solution of MZPES containing 207 mg

(equivalent to 150 mg of MZP base) per litre was prepared and an aliquot of a dilution of this solution, containing 0.45 micrograms of MZP, was added to residues of 0.45 micrograms of both MAP and MAP-Ac. Control urine (0.1 ml) was added to each tube and the tube contents were extracted and chromatographed. Using the mixed solvent the recovery of MZP, MAP and pyrimethamine exceeded 95% but the recovery of MAP-Ac was only 48% (Table 3.7). Because the recovery of pyrimethamine was similar to that of MZP and MAP the antimalarial was confirmed to be a suitable internal standard. Figure 3.4 is a representative calibration curve for MZP in urine.

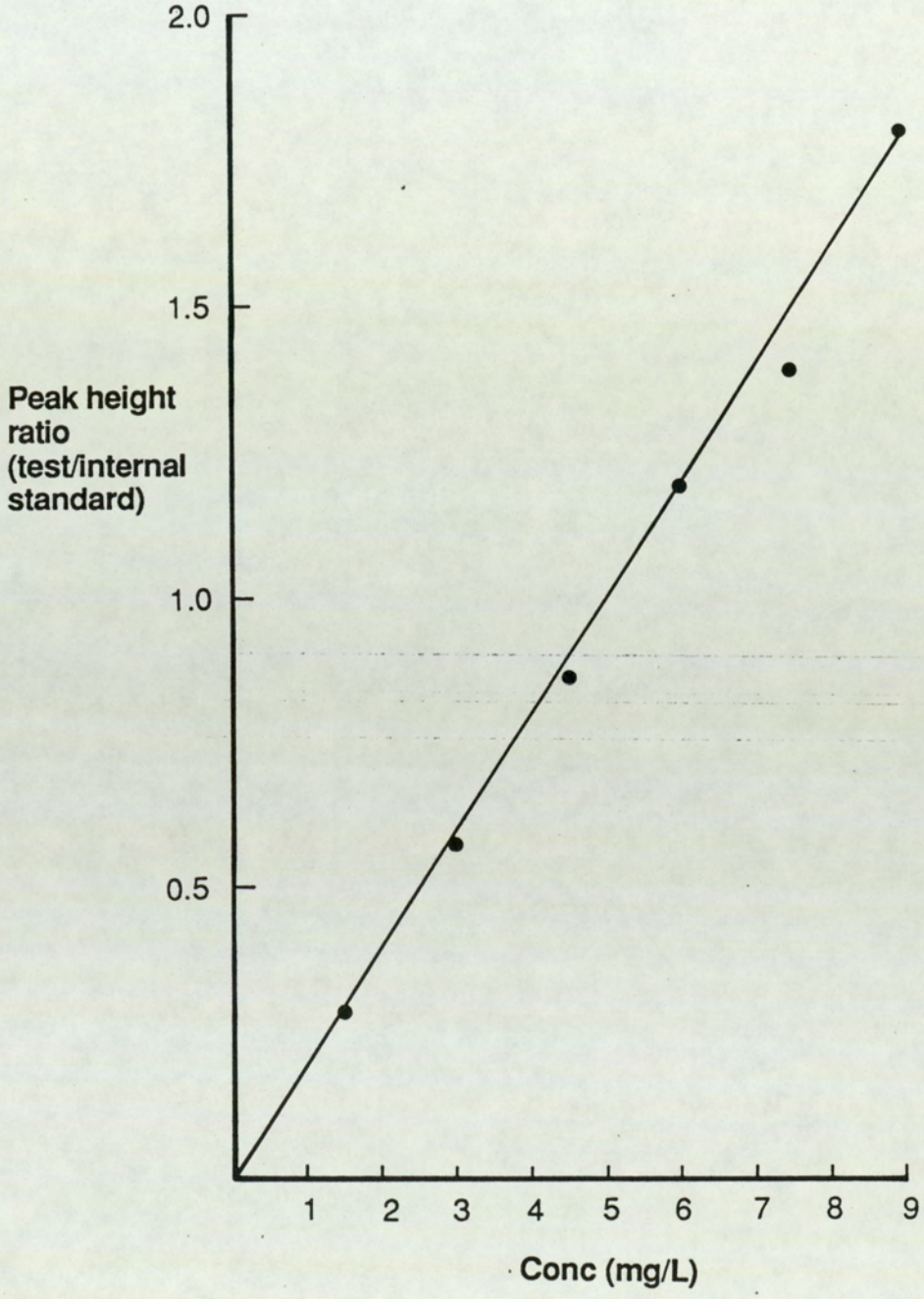
The extraction efficiency of the method for mouse plasma (Section 2.8) was determined. Using dichloroethane as extraction solvent the recovery of MZP was 75.1%, the recovery of pyrimethamine was 85.4% and the extraction of MAP-Ac was 100.1%. Using the mixed extraction solvent the extraction efficiency for MZP and pyrimethamine was improved (99.4% and 100.5% respectively), the recovery of MAP was 93.4% but the recovery of MAP-Ac was low, only 49.2%.

Representative chromatograms of control human plasma and of the plasma of an MZP-treated patient are shown in Figure 3.5. The former trace is virtually free from peaks due to endogenous compounds at the retention times of MAP-Ac, MAP, pyrimethamine and MZP. An endogenous peak appeared approximately 3.5 minutes after injection. A reagent blank run under identical conditions did not display any peaks.

The detection limit of the procedure for human plasma was ascertained by serially diluting a plasma solution of MZPES and assaying the dilutions with the LC3 detector at maximum sensitivity. The minimum concentration of MZP detectable with a signal-to-noise ratio of greater than 2 was 0.05 mg/L.

The recovery of MZP from faeces and liver was determined by spotting aqueous MZPES solution into control

Figure 3.4 Representative calibration curve for MZP in urine



1-Chlorobutane:dichloromethane 96:4 was used as extraction solvent

Figure 3.5 Chromatograms of human plasma extracts

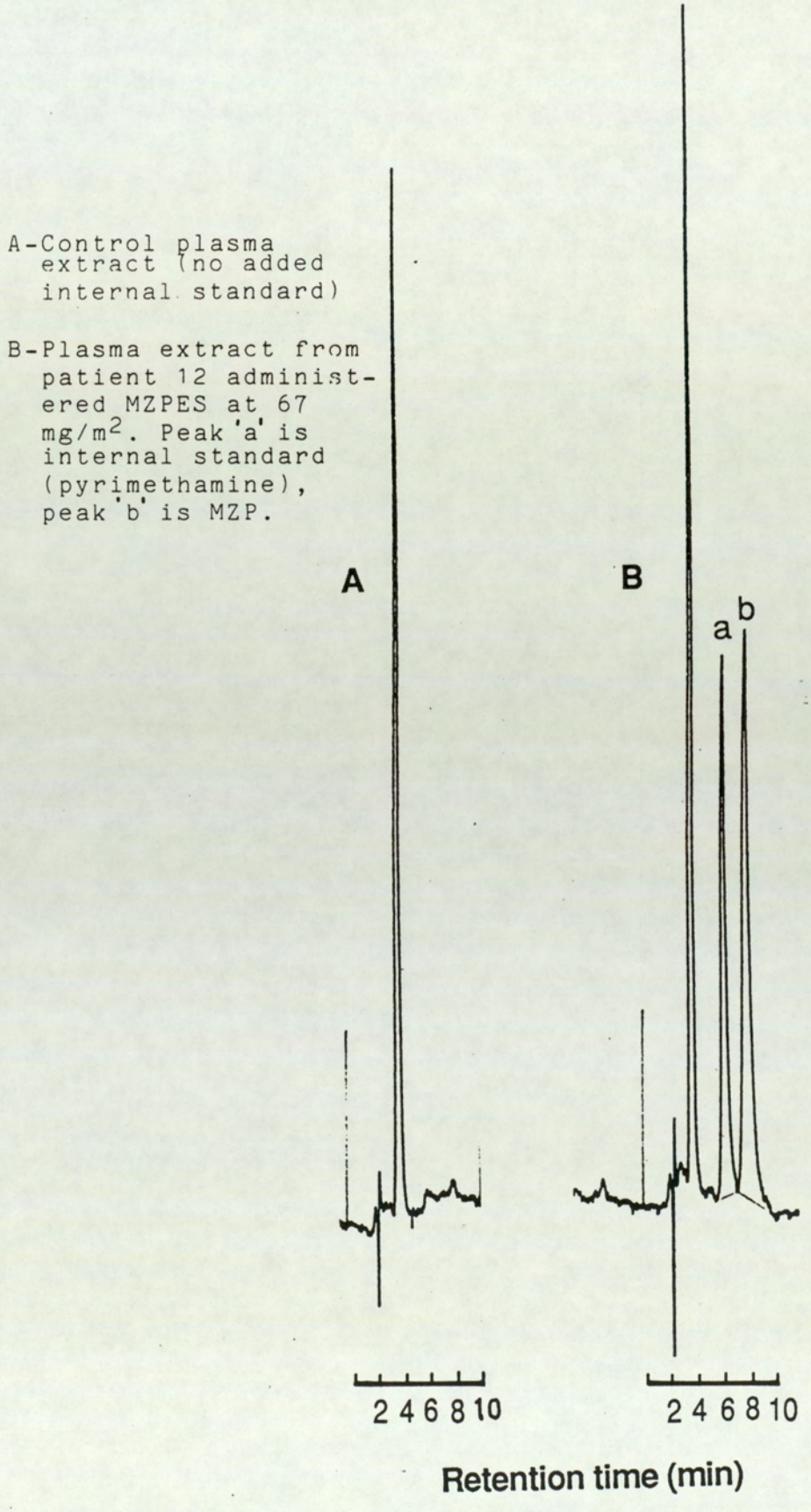


Table 3.8 Precision of analytical procedure for MZP in human plasma

	MZP concentration (mg/L)	CV (%)	n
Samples stood in Eppendorf tubes at -20°C and analysed over several months, each set of 5 on separate days. See Figure 3.6	0.224	8.2	5
	0.220	7.1	5
	0.215	6.0	5
	0.256	5.1	5
	0.237	5.5	5
Day-to-day precision (calculated from mean of each day's assays)		7.2	5
Samples stored in glass tubes at -20°C and duplicates analysed over several months on separate days. See Figure 3.7	0.987	6.2	54

rodent material. Recoveries were 95% at 6.0 mg/kg (faeces) and 90% at 22.5 mg/kg (liver). The procedures were validated for MAP and MAP-Ac by the method described for rat urine, with introduction of the standard material to the Griffiths tubes. Replicate analyses of individual faeces samples from MZP-treated rats demonstrated that the homogenization step achieved adequate distribution of MZP within faecal matter.

3.1.2 Assay precision

The reproducibility of the analytical procedure was determined using quality control plasma samples. The within-run CV at 0.25 mg/L did not exceed 8.2% and the day-to-day CV (calculated from the mean of each day's figures) was 7.2%. Quality control plasma containing MZP at 1.0 mg/L was assayed during the course of the human pharmacokinetic study. The grand mean concentration of MZP found in the quality control samples was 0.987 mg/L (n = 54) and the CV was 6.2%. The results of the assay reproducibility study are summarized in Table 3.8 and presented graphically in Figures 3.6 and 3.7.

Figure 3.6 Analysis of quality control plasma (MZP 0.25mg/L nominal) on 5 days over a period of several months. Stored in Eppendorf tubes at -20 degC

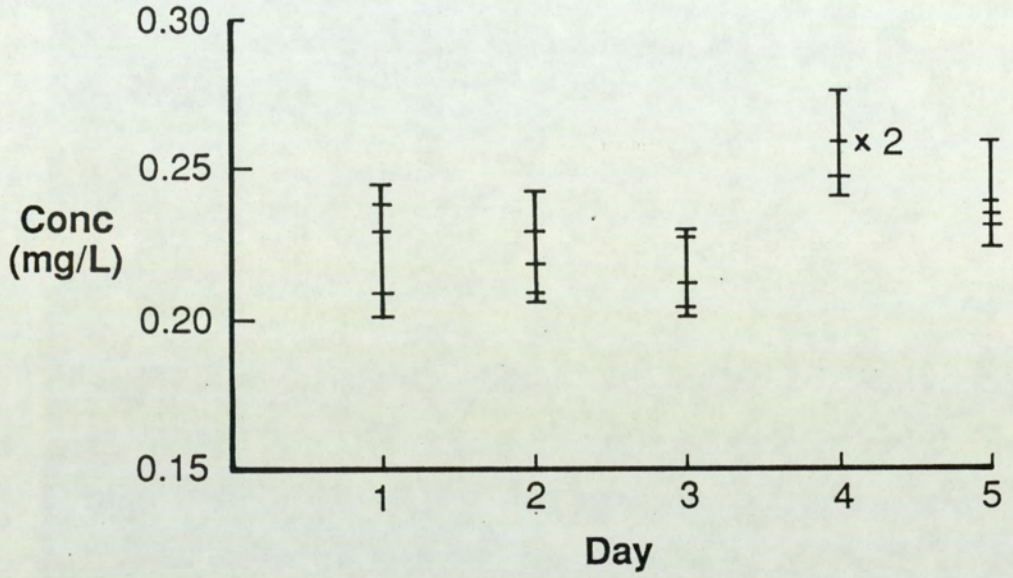
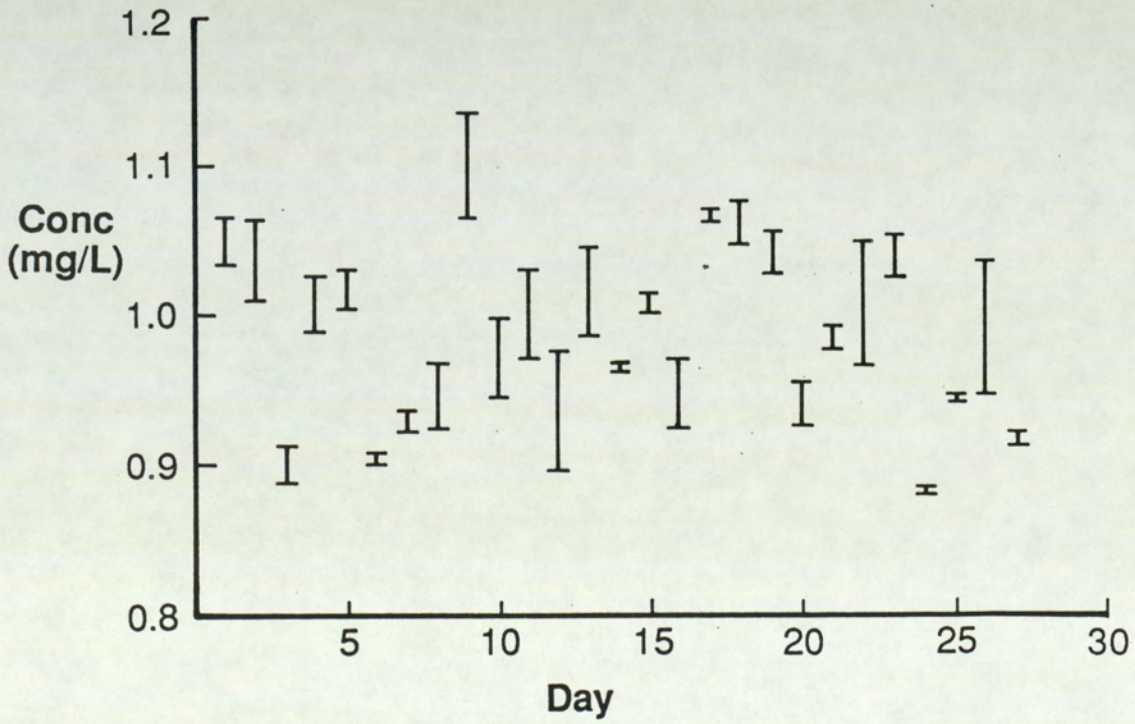


Figure 3.7 Analysis of quality control plasma (MZP 1.0mg/L nominal) on 27 days over a period of several months. Stored in glass tubes at -20 degC



A further investigation showed that there was no appreciable change in assay precision when dichloroethane was substituted for 1-chlorobutane:dichloromethane 96:4.

3.1.3 Stability of MAP-Ac in the extraction procedure

An experiment was conducted to determine whether MAP-Ac was stable under the conditions used for extraction of the 2,4-diaminopyrimidines. Control human urine and control urine spiked with MAP-Ac were analysed by the standard procedure (Section 2.7). Internal standard was omitted. The chromatograms differed only by the presence of a peak corresponding to MAP-Ac in the latter sample, thus the extraction process did not cause hydrolysis of MAP-Ac to MAP.

3.1.4 Discussion

The solvent of choice for extraction of MZP and MAP from fluids and tissues was 1-chlorobutane:dichloromethane 96:4. The recovery of MAP-Ac using this solvent was however rather low (about 50%) and was inferior to that achieved using 1,2-dichloroethane. The use of dichloromethane to extract amines may lead to the formation of artifacts, principally quaternary chloromethochloride salts (183), but the present work produced no evidence that such unwanted reactions occur with 2,4-diaminopyrimidine derivatives. Cavallito et al (75) homogenised tissue samples containing pyrimethamine, metoprine or etoprine in 2N lactic acid before basification and extraction and this solvent could be useful for dispersion of tissues containing MZP and its metabolites. The recovery of MAP-Ac may be improved by replacing the saline used in the present work with this solvent.

All the methods described separated MZP and MAP from endogenous material and allowed the discriminative measurement of the 2 compounds. The procedures permitted detection of MAP-Ac but the material was often incompletely resolved from early-running endogenous peaks from urine and faeces

samples. Chromatograms of the following pairs of control samples were virtually indistinguishable: rat and human urine; mouse and human plasma; rat urine and faeces; murine plasma and tissues. Calibration curves for MZP MAP and MAP-Ac in urine were linear to at least 9 mg/L. Linearity of measurement of these compounds was established to 300 mg/kg in faeces and to 45 mg/kg in tissues. Calibration curves constructed for the human pharmacokinetic study were linear to at least 35 mg of MZP per litre of plasma. In all cases correlation coefficients for fits to a straight line exceeded 0.99 and intercepts were not appreciably removed from zero. The linearity of calibration curves provided evidence that recovery was independent of concentration within working ranges.

The analytical methods described were robust, accurate, reasonably precise and sensitive. Small sample volumes or weights were required and a concentration factor of up to 4.5 could be introduced. Retention times were short and each overall procedure was rapid. Retention times were very reproducible and a single Partisil 10 ODS 2 column was used for many hundreds of measurements with virtually no deterioration in performance.

3.2 Thin-layer chromatography

The general procedure described in Section 2.2.3 was used in attempts to separate MZP, MAP, MAP-Ac, MZP-N-1-oxide and MZP-N-3-oxide. Table 3.9 present Rf values for these compounds using a variety of solvent systems obtained from the literature and modified as indicated. The best separations were achieved using chloroform:methanol 7:3 or butan-1-ol:water:acetic acid 12:3:1. Spots showed marginally higher resolution using the latter system.

Visualization at 254 nm was superior to that at 375 nm. The sensitivity of detection was determined by serially diluting the stock 0.1% w/v solutions to 1 in 100 with methanol (the stock 0.01% w/v solution of

Table 3.9 Rf values for MZP and some related compounds

Solvent system	MZP	MAP	MAP-Ac	MZP-N-3-oxide	MZP-N-1-oxide	Reference
Chloroform:methanol 7:3	·49	·29	·37	·43	·14	134
Chloroform:methanol 1:1	·51	·37	·43	·43	·17	-
Chloroform:methanol 9:1	·19	·60	·70	·13	·20	-
Butan-1-ol:water: acetic acid 6:1:1	·43	·40	·35	·50	36	150
Butan-1-ol:water: acetic acid 12:3:1	·45	·40	·34	·50	·28	-
Butan-1-ol-water: acetic acid 12:1:3	·41	·36	·28	·50	·32	-
Butan-1-ol-water: acetic acid 11:3:2	·60	·57	·51	·65	·51	-
Butan-1-ol:water: acetic acid 13:1:2	·36	·31	·24	·44	·25	-
Butan-1-ol:water: acetic acid:pyridine 6:1:1:1	·58	·55	·53	·57	·45	150
Chloroform:ethanol acetic acid 90:10:5	·17	·07	·05	·48	·13	133
* Toluene:ethyl acetate diethylamine 7:2:1	·10	·04	·03	·03	0	137
Propan-2-ol:methanol: ammonia 50:40:1	·50	·48	·48	·42	·15	75
Propan-2-ol:chloroform: ammonia 20:25:2	·73	·64	·65	·61	·23	150
Propan-2-ol:chloroform: ammonia 20:25:0·01	·35	·15	·07	·19	·02	141
Butan-1-ol:propan-2-ol: water:ammonia 10:5:4:1	·66	·65	·63	·61	·37	150

* Toluene substituted for benzene used by authors

Adsorbent Kieselgel 60 F 254 (Merck no.5735) 0.2 mm thick.

MZP-N-1-oxide was diluted to 1 in 10) and applying 4 x 5 microlitres of each solution, with drying between each application. A loading of 400 ng represented the minimum amount of each material that could be seen at 254 nm.

3.2.1 Discussion

In the analysis of a drug in biological material the chromatographic behaviour of co-extracted endogenous material cannot be foreseen and it is necessary to evaluate a variety of chromatographic systems and determine which one produces the best isolation of drug and metabolites. Although chloroform:methanol 7:3 and butan-1-ol:water: acetic acid 12:3:1 achieved the best separation of MZP, MAP, MAP-Ac, MZP-N-1-oxide and MZP-N-3-oxide on silica gel plates other systems could be of value. For example, propan-2-ol:chloroform:ammonia 20:25:2 did not separate MAP and MAP-Ac but did resolve MZP, MZP-N-1-oxide and MZP-N-3-oxide and the Rf values of these 3 compounds differed appreciably from those found using the afore-mentioned solvent systems.

The limit of detection of MZP by TLC (400 ng) was inferior to that achieved using HPLC (2 ng injected, signal-to-noise ratio = 2).

3.3 Analysis of MZP-N-oxides

The procedure for MZP, MAP and MAP-Ac in urine (Section 2.7) was evaluated as a method of analysis for MZP-N-1-oxide and MZP-N-3-oxide. 1-chlorobutane:dichloromethane 96:4 was used as extraction solvent and methanol:phosphate (0.02 M, pH 3.0) 75:25, containing 0.02 M SDS, was the mobile phase employed. Detection was at 247 nm. None of the N-1-oxide and 67% of the N-3-oxide were recovered from urine seeded at 4.5 mg/L with each material.

Hubbell et al (150) detected the N-oxides of metoprine and pyrimethamine in rat urine by saturating samples from

drug-treated animals with ammonium sulphate and extracting with ether:ethanol 3:1 v/v. In the present study this procedure was evaluated as a means of extracting the N-oxides of MZP from biological material. The recovery of MAP-Ac using 1-chlorobutane:dichloromethane 96:4 as extraction solvent was low (Table 3.7) thus this material was examined alongside the N-oxides in Hubbell's procedure. Control urine was spiked with each material at 4.5 mg/L, extracted and the organic layer was removed, dried and evaporated. The residue was examined by HPLC as before. Unspiked urine was also examined by the same procedure. The recovery of MZP-N-3-oxide was 70% but an endogenous peak interfered with the MZP-N-1-oxide and MAP-Ac peaks.

In a further attempt to develop a method of analysis for the MZP-N-oxides and MAP-Ac both urine and plasma were spiked with the materials, the samples were extracted with 1-chlorobutane:dichloromethane 96:4 (after addition of alkali) or with ether:ethanol 3:1 v/v (after saturation with ammonium sulphate) and the extracts were examined by TLC. Spiked control samples were examined alongside test samples. Each final residue was reconstituted in methanol for application to the TLC sheet. The TLC systems were as described in Sections 2.2.3 and 3.2. Chloroform: methanol 7:3 and butan-1-ol:water:acetic acid 12:3:1 were the solvent systems employed. The only procedure of any value was 1-chlorobutane:dichloromethane 96:4 extraction with TLC analysis using butan-1-ol:water:acetic acid 12:3:1 as solvent, but even with this system separation of MZP-N-1-oxide from endogenous materials was incomplete. Endogenous interference was overwhelming using all other systems.

Development of an assay for the MZP-N-oxides was not pursued further. Hubbell et al (150) isolated the N-oxides of pyrimethamine and metoprine from rat urine by chromatographing ether:ethanol 3:1 extracts on fluorisil columns, using increasing concentrations of methanol in chloroform as eluant, and this procedure may be of use for isolation of the N-oxides of MZP. Levin et al (153)

precipitated endogenous material in rat plasma and tissue with a methanol:phosphate buffer mixture and assayed metoprine in the supernatant liquid by HPLC. This work also may provide an avenue for analysis of MZP metabolites in fluids and tissues.

3.4 Tentative identification of MZP and MAP in samples by measurement of ultraviolet absorbance ratios

Preliminary analyses of the urine and faeces of rats dosed with MZP, and of the tissues of MZP-treated mice, revealed chromatographic peaks corresponding in retention times to MZP and MAP. Very low levels of a compound corresponding to MAP-Ac were detected in some urine and faeces samples, but these peaks were poorly separated from unknown shorter retention time peaks. In order to confirm the identity of the "MZP", "MAP" and "MAP-Ac" peaks absorbance ratio measurements (184) were made. The identity and purity of a component appearing as a peak in HPLC can be assured by measuring its ultraviolet absorbance at several wavelengths. Absorbance ratios are calculated and compared with the values obtained for an injected standard. If the ratios are the same the identity of the test peak is confirmed. If the ratios differ the presence of unresolved components can be suspected or the identity of the test peak is in question.

Spectroscopy (Section 3.1.1) demonstrated that MZP, MAP and MAP-Ac have appreciable absorbance at 247, 257, 272 and 287 nm in acidic solution. A rat urine sample was selected for evaluation. A solution of MZP, MAP and MAP-Ac in mobile phase was prepared in which the concentration of each component approximated to those found in the reconstituted final extract when the sample was analysed by the standard procedure (Section 2.7). Ten aliquots of the sample were extracted with dichloroethane by this method. Internal standard was omitted. The reconstituted extracts were pooled and stored in a small stoppered glass tube. The synthetic solution and the

pool were chromatographed by the standard procedure, each in triplicate, with the LC3 detector set at each of the predetermined wavelengths in turn. The height of each standard peak, and of each test peak correspondingly closely in retention time to a standard peak, was measured. Peak heights were averaged and for each peak the ratio of the height at each wavelength to the height at every other wavelength was calculated. Absorbance ratio measurements were also made on a rat faeces sample and a mouse liver sample.

Absorbance ratios and retention times for the peaks observed in extracts of the urine, faeces and liver samples, together with data for the standard MZP, MAP and MAP-Ac peaks, are presented in Table 3.10. Ratios for standard and proposed MZP peaks generally diverged by 0 to 20% but deviations of up to 34% (in liver) were seen. Divergences between ratios for standard and proposed MAP peaks were within the range 0 to 14%. Absorbance ratios for standard MAP-Ac differed from those for proposed MAP-Ac in extracts by 11 to 64%.

3.4.1 Discussion

If the unknown compound is not identical with the standard then absorbance ratios are unlikely to match fortuitously with 3 or more well-separated wavelengths (184). Combining absorbance ratio measurements with retention times gives added certainty in peak identification (185) and in the present work the match of retention times between standard and test peaks was reasonable. The data in Table 3.10 provide evidence that the peaks in question in urine, faeces and liver chromatograms were MZP and MAP and that co-eluting components were absent. Identification of MAP-Ac in urine and faeces was less certain.

Variation of background absorbance over the wavelength range covered will introduce error into this method of identification. Such variation is unlikely to occur in synthetic solutions but may be considerable in biological

Table 3.10 Retention times and absorbance ratios for MZP, MAP and MAP-Ac and for test materials in rat urine, rat faeces and mouse liver

Sample	Peak	rt	Absorbance ratios $\frac{A_{257}}{A_{272}}$	$\frac{247}{287}$	$\frac{247}{272}$	$\frac{272}{287}$	$\frac{257}{287}$	$\frac{247}{257}$
Rat urine	a	4.10	1.51	2.91	2.96	0.98	1.48	1.97
	b	5.10	1.34	2.77	2.76	1.01	1.34	2.06
	d	8.50	1.45	2.81	1.77	1.58	2.30	1.22
Standard MAP-Ac		4.13	1.69	3.64	2.66	1.37	2.32	1.57
MAP		5.17	1.37	2.76	2.63	1.05	1.44	1.92
MZP		8.82	1.51	3.02	1.64	1.85	2.79	1.08
Rat faeces	e	4.31	1.67	3.00	3.41	0.88	1.47	2.04
	f	5.37	1.63	3.05	3.11	0.98	1.60	1.91
	g	9.01	1.67	2.91	1.84	1.59	2.65	1.10
Standard MAP-Ac		4.13	1.86	3.69	2.84	1.30	2.41	1.53
MAP		5.17	1.50	2.81	2.81	1.00	1.50	1.87
MZP		8.82	1.59	2.90	1.85	1.57	2.49	1.16
Mouse liver	h	3.91	1.53	2.37	2.59	0.91	1.40	1.70
	i	5.77	1.72	2.46	1.97	1.25	2.15	1.15
Standard MAP		3.91	1.49	2.69	2.58	1.04	1.56	1.73
MZP		5.77	1.69	3.13	1.86	1.68	2.84	1.10

Column Partisil 10 ODS 2, mobile phase methanol:phosphate (0.02 M, pH 3.0) 79:21 (urine and faeces) 83:17 (liver) containing 0.02 M SDS, flowrate 1.4 ml/min

rt - minutes

Rat urine - 24 to 48 hour, MZP dosage 50 mg/kg IP

Rat faeces - 0 to 21 hour, MZP dosage 100 mg/kg IP

Mouse liver - 2 hour sample, MZP dosage 20 mg/kg IP

Peaks a, b and d (rat urine) refer to Figure 3.3. Peaks e, f, g (rat faeces) h and i (mouse liver) were those corresponding most closely to standard peaks.

extracts. The proposed MAP-Ac peak was poorly separated from an earlier-running component and this was probably an additional source of error. Divergences of absorbance ratios between standard and unknown peaks were also due to employment of absolute peak height measurements. Measurements were not made relative to an internal standard and error in peak height was translated into a compound error when ratios were calculated. CVs of replicate peak height measurements at specific wavelengths did not exceed 4% however.

3.5 Stability of MZP and MAP in frozen samples

Evaluated samples of plasma and liver from MZP-treated mice were re-assayed after storage at -20°C for several months. Samples of urine and faeces from rats dosed with MZP were stored at -20°C and re-analysed some months after the initial analyses. Plasma samples from the human pharmacokinetic study were assessed in a similar fashion. Quality control plasma was assayed, stored at -20°C in Sterilin vials and re-assayed in order to determine whether there was any change in MZP content. The results of these investigations are summarised in Table 3.11.

Mouse plasma showed no appreciable change in MZP content after 52 weeks at -20°C. Human plasma lost maximally 10.9% MZP after storage deep-frozen for 65 weeks and quality control plasma apparently lost up to 13.6% MZP after storage under the same conditions for 10 weeks. The rat urine sample showed no change in concentrations of MZP and MAP after storage for 28 weeks but beyond this time losses of both components were apparent. MZP and MAP were stable in rat faeces for no more than 26 weeks at -20°C. MZP was very labile in samples of liver from drug-treated mice. After storage for 26 weeks at -20°C 3 samples showed apparent loss of MZP within the range 27.5 to 55.0% and increases in MAP concentrations of 52.5 to 58.0%, relative to the initial assay figures. Post-storage samples of plasma, urine, faeces and liver developed no spurious peaks.

Table 3.11 Stability of MZP in plasma and MZP and MAP in liver, urine and faeces stored at -20°C

	Storage time (weeks)	Mean assay (mg/L or mg/kg, n = 2) Initial MZP	Initial MAP	Final MZP	Final MAP	Change (%) MZP	Change (%) MAP
Mouse plasma	52	1.910	-	1.930	-	+ 1.1	-
	52	2.150		2.160		+ 0.5	
Human plasma	78	0.200		0.189		- 5.5	-
	65	0.726		0.647		- 10.9	
	65	0.324		0.321		- 0.9	
Quality control plasma	10	0.940		0.930		- 1.1	
	10	0.986		0.852		- 13.6	
	10	1.011		0.929		- 8.1	
Rat urine	28	5.70	3.00	5.70	3.00	0	0
Rat faeces	26	64.20	103.50	67.00	104.50	+ 4.4	+ 1.0
Mouse Liver	26	38.90	19.90	28.30	30.40	- 27.5	+ 52.8
	26	28.70	28.80	12.90	45.50	- 55.0	+ 58.0
	26	17.60	21.70	11.10	33.10	- 36.9	+ 52.5

Mouse plasma was stored in Eppendorf tubes. All other samples were stored in Sterilin vials. Initial figures for quality control plasma were obtained immediately before transference of samples to vials.

3.5.1 Discussion

The data for quality control samples and for samples from MZP-treated patients suggest that MZP degrades slowly in plasma. The drug may be adsorbed onto the surface of Sterilin vials upon storage. The CV of the analytical procedure was however 6.2% at 1.0 mg/L (Table 3.8) and there was wide variation in apparent losses of MZP in both individual quality control plasma samples (1.1, 8.1 and 13.6%) and individual patient samples (0.9, 5.5 and 10.9%). These variations would appear to be the result of analytical imprecision and examination of many more samples would be necessary to confirm loss of MZP from plasma stored in plastic vials. The data for patient and mouse plasma provide some reassurance that losses of MZP upon storage at -20°C were not significant.

MZP and MAP were stable in rat urine and faeces for acceptable periods of time.

3.6 Stability of MZP, MAP and MAP-Ac in plasma and urine at 37°C

Figure 3.8 represents the concentrations of MZP, MAP and MAP-Ac found in spiked plasma incubated at 37°C for 11 hours and Figure 3.9 is the corresponding graph for urine.

In both plasma and urine the apparent concentration of each material increased with time. Weight loss from the tared tubes did not exceed 0.25% over 11 hours thus evaporation was not the cause of the observed increasing levels. Chromatographs of pre- and post-incubation samples showed no obvious differences and the control samples developed no spurious peaks on incubation. After 11 hours the mean concentrations of MZP in plasma and urine had risen by 1.7 and 1.2% respectively and amongst all measurements the highest apparent increase in concentration (MAP in urine) was 5.4%.

The concentrations of MZP and MAP found in both

Figure 3.8 Stability of MZP, MAP and MAP-Ac in plasma at 37 degC

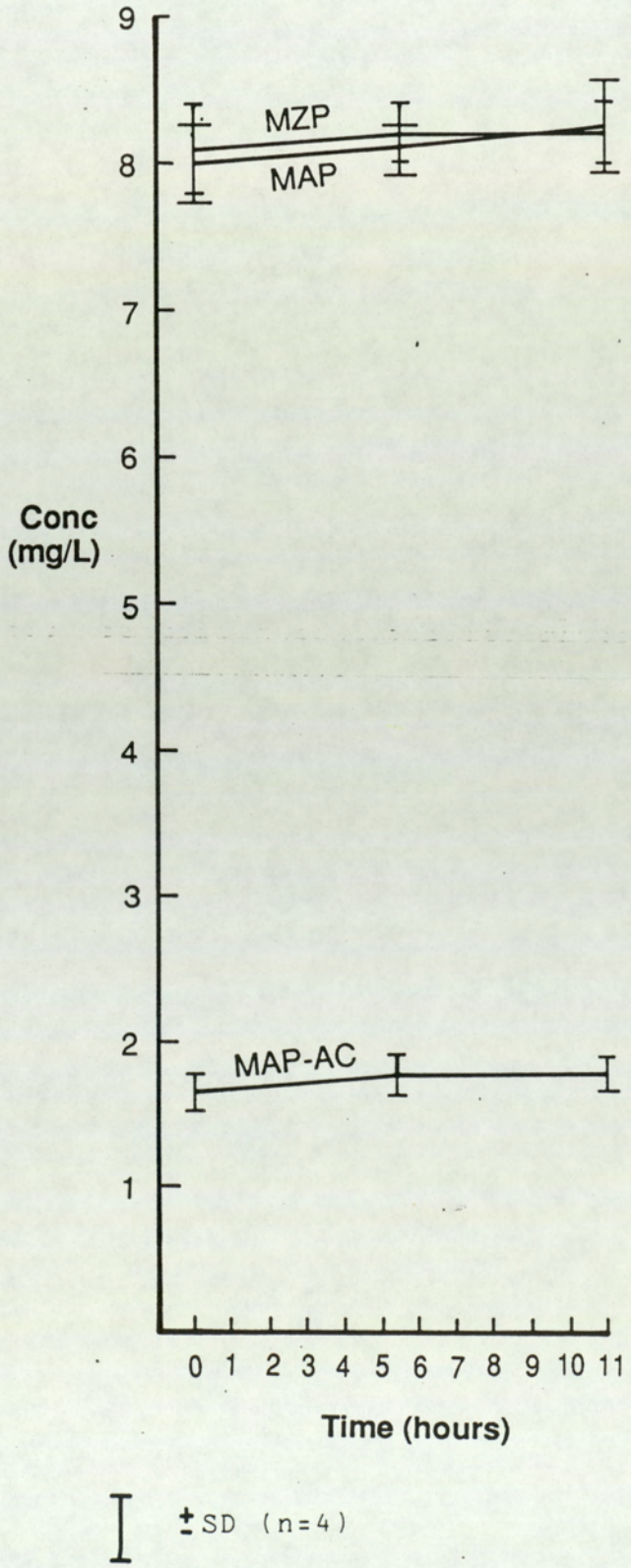
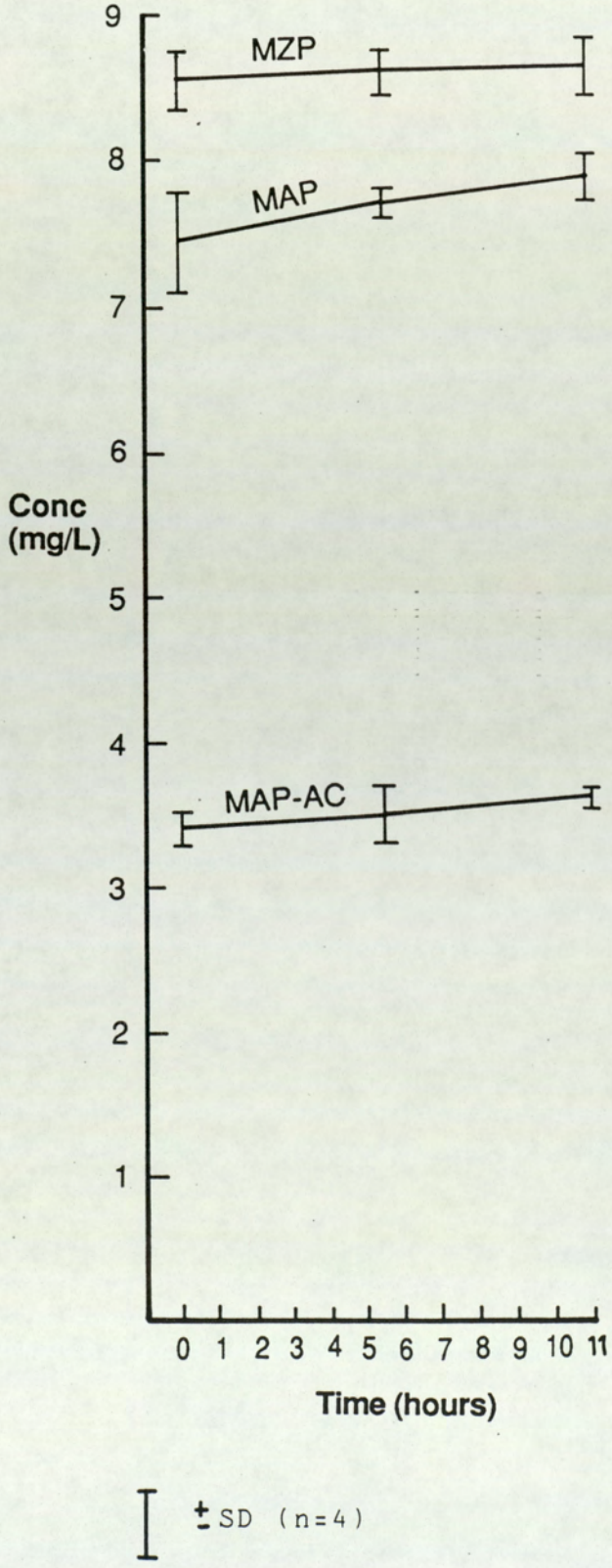


Figure 3.9 Stability of MZP, MAP and MAP-Ac in urine at 37 degC



plasma and urine were close to the theoretical level (9.0 mg/L) but MAP-Ac was poorly soluble in these fluids, reaching concentrations of only 3.5 and 1.75 mg/L in urine and plasma respectively.

3.6.1 Discussion

Chemical change would not appreciably contribute to pharmacokinetic parameters for MZP, MAP and MAP-Ac measured *in vivo*. Evident increases in concentration of MZP, MAP and MAP-Ac upon incubation in plasma and urine for 11 hours may have been due to formation of small amounts of reaction products showing the same retention times as the parent compounds but higher UV absorbance.

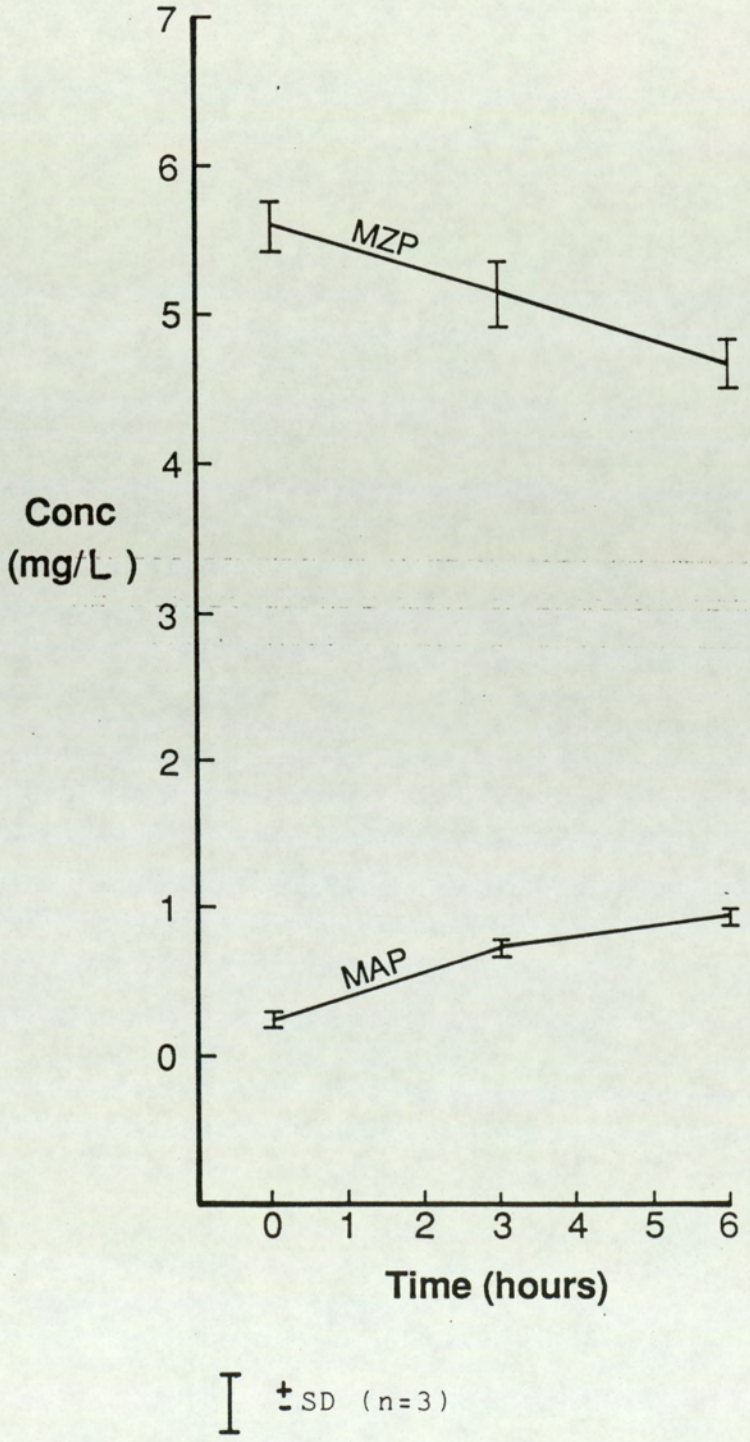
3.7 Reaction of MZP with some cellular reducing agents

There was no production of MAP in the absence of reductant at either pH or at pH 7.4 with a 55-fold molar excess of glutathione. MAP formation was evident at both pH values with a 550-fold molar excess of glutathione or with a 1400-fold molar excess of cysteine. At pH 5.0 MAP was produced in the presence of a 55-fold molar excess of glutathione. The greatest fall in MZP concentration over 6 hours (apparently 15%) and the highest production of MAP was seen at pH 7.4 with cysteine present (Figure 3.10). All chromatograms displayed peaks corresponding only to MZP and, where described, MAP. Evaporation losses over 6 hours were negligible.

3.7.1 Discussion

Glutathione is a naturally-occurring tripeptide which takes part in detoxification mechanisms in the body (186) and is the major low molecular weight thiol compound in the animal cell (187). L-cysteine is a precursor of glutathione (188). The normal concentration of glutathione in whole blood is 354 mg/L (189) thus the agent was incorporated into reaction mixtures at this level

Figure 3.10 Stability of MZP with a 1400-fold molar excess of L-cysteine in buffer pH 7.4 at 37 degC



and at a tenfold higher concentration. The concentration of L-cysteine employed in the experiments was 3.54 g/L. A preliminary analysis of MZP in buffer pH 9 gave low recovery, probably because of poor solubility or instability of the drug in alkali, thus a mildly alkaline buffer (plasma pH, 7.4) and an acidic buffer (pH 5.0) were used for the subsequent investigations.

The experiments demonstrated that, under the conditions employed, the reduction of MZP to MAP by reduced glutathione or L-cysteine is not facile. The work could be extended by employment of buffers covering a wider range of pH values, but this may represent a departure from physiological conditions.

3.8 Degradation of MZP on incubation with whole mouse liver

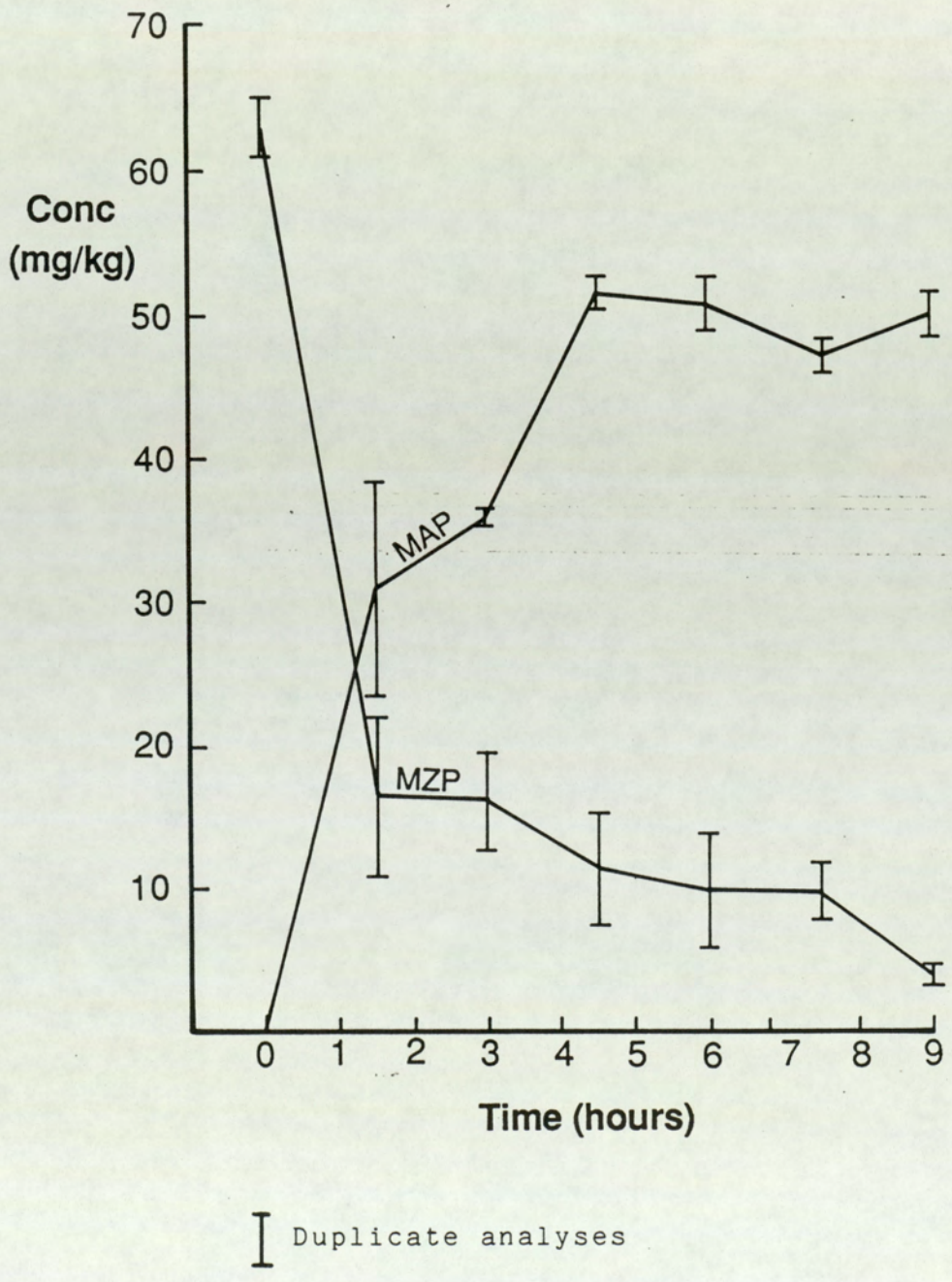
Figure 3.11 represents the time-course of degradation of MZP to MAP in liver over 9 hours at 37°C.

MAP formation was evident after incubation for 1.5 hours and the concentration of the metabolite ascended with time to reach a maximum after 4.5 hours. MZP degradation continued to 9.0 hours, which suggested that the drug was converted to other metabolites as well as to MAP. The analytical method was not capable of detecting these other compounds. Chromatograms of pre- and post-incubation samples differed only with respect to the sizes of the MZP and MAP peaks and the control sample developed no spurious peaks on incubation.

3.8.1 Discussion

The experiment showed that whole mouse liver provides a milieu for the facile reduction of MZP to MAP. The storage trial (Section 3.5) also demonstrated that MZP is readily converted to MAP in liver. In the incubation experiment MZP degradation was apparently not solely conversion to MAP thus it was not possible to calculate the

Figure 3.11 Time-course of degradation of MZP to MAP in whole mouse liver at 37 degC



mass balance of MZP degradation to MAP formation.

There was no appreciable degradation of MZP upon incubation in plasma or urine over 11 hours (Section 3.6) or upon storage of plasma, urine or faeces from MZP-treated animals at -20°C for several months (Section 3.5). The inference is that the enzyme or other agent in liver which can degrade MZP is absent from these other materials.

3.9 MZP excretion balance study in the rat

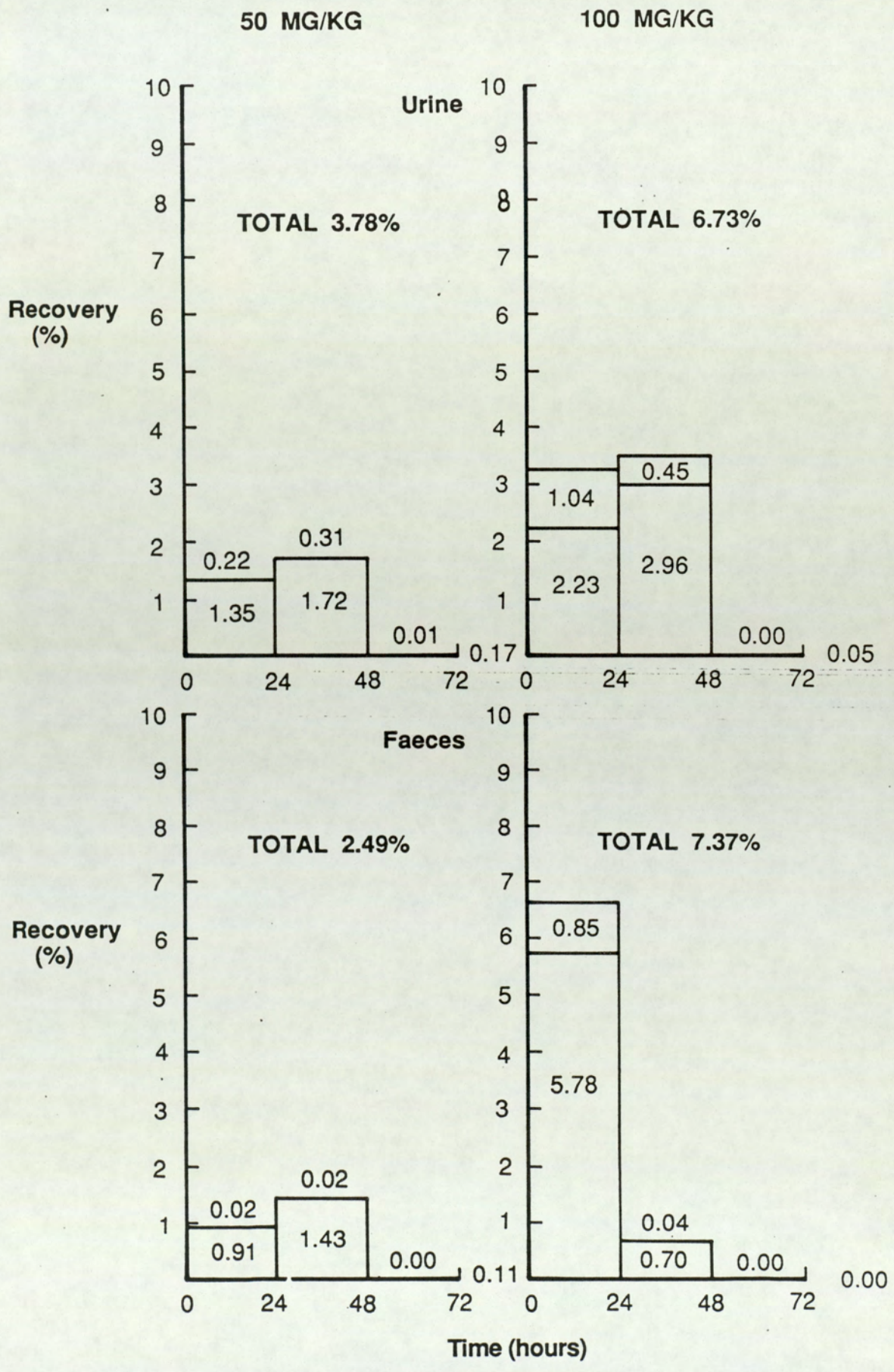
Assays were completed within 8 weeks from the time of collection of samples. Storage trials (Section 3.5) demonstrated that MZP and MAP were stable in deep-frozen urine and faeces for 26 weeks.

Many samples of both urine and faeces from MZP-treated rats showed early-running chromatographic peaks which were larger and more numerous than those seen in control samples. These test sample peaks must therefore have been biotransformation products of MZP. They were however not reproducible in size or retention time even upon repeat analysis of individual samples.

The rats employed in the study showed no appreciable weight changes during the course of the experiments. In each study the weights of MZP and MAP found in urine and faeces were converted to percentages of the MZP dose administered. MAP recovered was assumed to be equivalent, on a weight basis, to MZP administered. A detailed breakdown of the urine and faeces levels of MZP and MAP found at each dose, together with the percentage recovery of each component, is given in Appendix 1. Figure 3.12 presents the mean results as histograms.

For the rats dosed at 50 mg/kg the mean recovery was 3.78% in the urine (MZP 0.54% and MAP 3.24%) and 2.49% in the faeces (MZP 0.04% and MAP 2.45%). The mean recovery from the rats dosed at 100 mg/kg was 6.73% in the urine (MZP 1.49% and MAP 5.24%) and 7.37% in the faeces (MZP 0.89% and MAP 6.48%). MAP excretion exceeded that

Figure 3.12 Recovery of MZP and MAP in the urine and faeces of rats after IP administration of MZP



MZP upper, MAP lower

of MZP. There was no appreciable difference between the amounts of drug plus metabolite excreted in urine and faeces. A decay pattern was not clearly evident over 48 or 72 hours.

3.9.1 Discussion

After IP administration of MZP in the rat the cumulative amount of intact drug excreted in urine and faeces over 72 hours did not exceed 2.38% of the dose. Biodegradation of MZP to MAP was considerable but the maximum amount of drug plus metabolite that could be recovered was 14.1% of the MZP administered. The low recovery and extensive metabolism of MZP is in accordance with the results of excretion studies on other lipophilic antifolates. Cavallito et al (75) reported that less than 5% of the metoprine given to rats (2.8 mg/kg PO) was excreted intact in the urine over 5 days. Pyrimethamine was excreted in the urine and faeces of rats (150) and mice (174) primarily as biotransformation products; excretion was prolonged for up to 7 days (174). DAMP was eliminated slowly in the urine and faeces of the rat both as unchanged drug and metabolites (19).

The low recovery of MZP may have been due to precipitation at the injection site, accumulation or extensive metabolism. The analytical procedure was designed to measure only unconjugated MZP, MAP and MAP-Ac, but published work (144,150,156,174) has shown that biotransformation of metoprine and pyrimethamine is considerable and it is likely that MZP is converted in vivo to a wide variety of metabolites.

3.10 MZP murine pharmacokinetics

3.10.1 Plasma pharmacokinetics

Analyses were completed within 2 weeks from the time of collection of plasma samples; assays after storage (Section 3.5) demonstrated that MZP was stable in mouse

Table 3.12 MZP plasma pharmacokinetic data in mice after IP administration

Dosage (mg/kg)	10	20
T_{max} (hr)	0.25	0.25
Cp_{max} (mg/L)	2.60	7.67
Distribution $t_{\frac{1}{2}}$ (hr)	0.59	0.64
Elimination $t_{\frac{1}{2}}$ (hr)	3.04	4.58
V_d (ml)	101	87

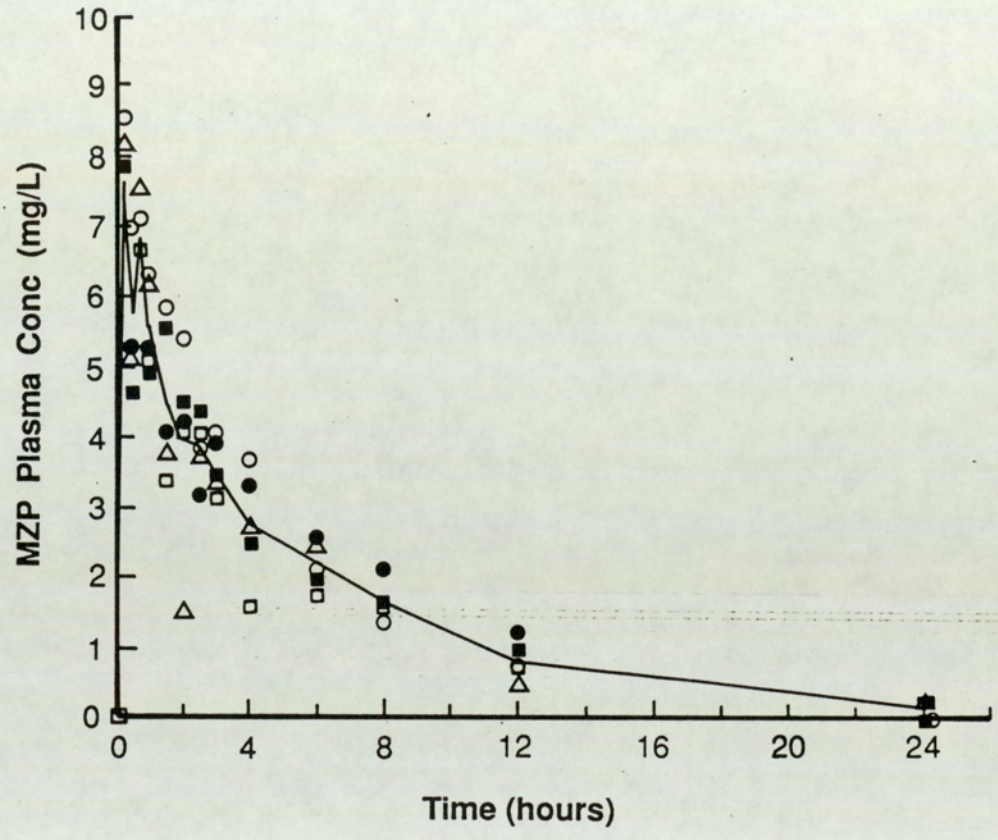
Values for V_d were calculated on the basis of a 25 g mouse weight

plasma at -20°C for at least 52 weeks. A quality control sample was analysed after each 5 test samples and results from a series of test plasmas were accepted if results for the quality control samples lay within the range ± 15% of predetermined values.

Only MZP was detected in mouse plasma; MAP or MAP-Ac were not evident at any time-point. The results obtained for the dose of 20 mg/kg IP are presented in Figure 3.13. The line represents a plot of the mean arithmetic plasma concentrations of MZP against time and the individual assay results for mice at each time-point are indicated. A detailed breakdown of the plasma levels of MZP found at each dose (10 and 20 mg/kg IP), together with values for mean and standard deviation are tabulated in Appendix 2.

In each case MZP was rapidly absorbed into the blood, with peak plasma concentrations (Cp_{max}) of 2.60 mg/L (10 mg/kg dosage) and 7.67 mg/L (20 mg/kg dosage) reached 15 minutes after injection. After 12 hours plasma MZP levels had fallen to 0.15 mg/L (10 mg/kg dosage) and 0.82 mg/L (20 mg/kg dosage). Pharmacokinetic parameters for the murine plasma data were estimated by assuming a 2-compartment model and data are presented in Table 3.12.

Figure 3.13 MZP plasma pharmacokinetics in mice dosed at 20 mg/kg IP



5 mice were sampled at each time point

MZP showed distribution half-lives of 0.59 and 0.64 hours at 10 and 20 mg/kg IP dosage respectively. Plasma elimination half-lives were 3.04 hours (10 mg/kg dosage) and 4.58 hours (20 mg/kg dosage). Apparent V_ds were 101 ml (10 mg/kg dosage) and 87 ml (20 mg/kg dosage).

3.10.2 Oral bioavailability

Mice were treated with MZP PO and the plasma profile was compared with that previously obtained following IP administration. In both cases the drug was formulated as aqueous MZPES solution. The resulting pharmacokinetic plots are presented in Figure 3.14. The lines represent plots of the mean arithmetic plasma concentrations of MZP against time. Tabulated details of the individual plasma levels of MZP determined after PO administration, together with values for mean and standard deviation, are presented in Appendix 2.

The Cp_{max} of MZP after PO dosage (2.07 mg/L) was lower than that achieved after IP dosage at 10 mg/kg (2.60 mg/L) and was reached 1 hour after administration. AUC_{trap} values (0 to 24 hr) were 13.43 and 10.88 mgL/hr by the oral and IP routes respectively. The oral bioavailability of MZP was thus 13.43/10.88 = 1.23.

The elimination half-life of MZP following oral administration in the mouse at 10 mg/kg was 4.28 hours, which was slightly higher than the value seen previously following IP administration at the same dosage level.

3.10.3 Tissue pharmacokinetics

Both MZP and MAP were detected in the liver, brain, lung, heart and kidney of mice after IP dosage with MZP at 20 mg/kg. Chromatograms of some kidney and heart extracts also showed very low levels of a material corresponding in retention time to MAP-Ac. The results of the study on brain tissue are presented as graphs in Figures 3.15 (MZP) and 3.16 (MAP). The lines represent plots of the mean arithmetic brain concentrations of MZP and

Figure 3.14 MZP plasma pharmacokinetics in mice dosed at 10 mg/kg IP and PO

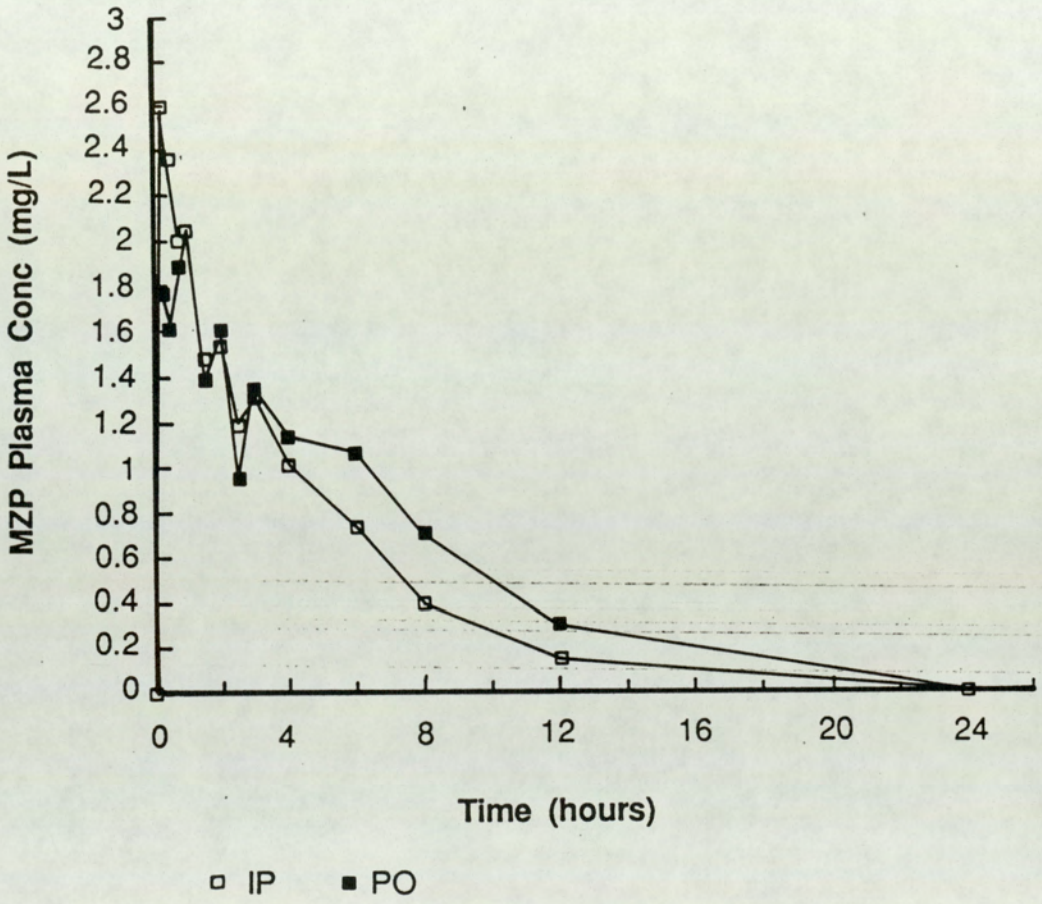


Figure 3.15 MZP brain and plasma concentrations versus time in mice dosed at 20 mg/kg IP

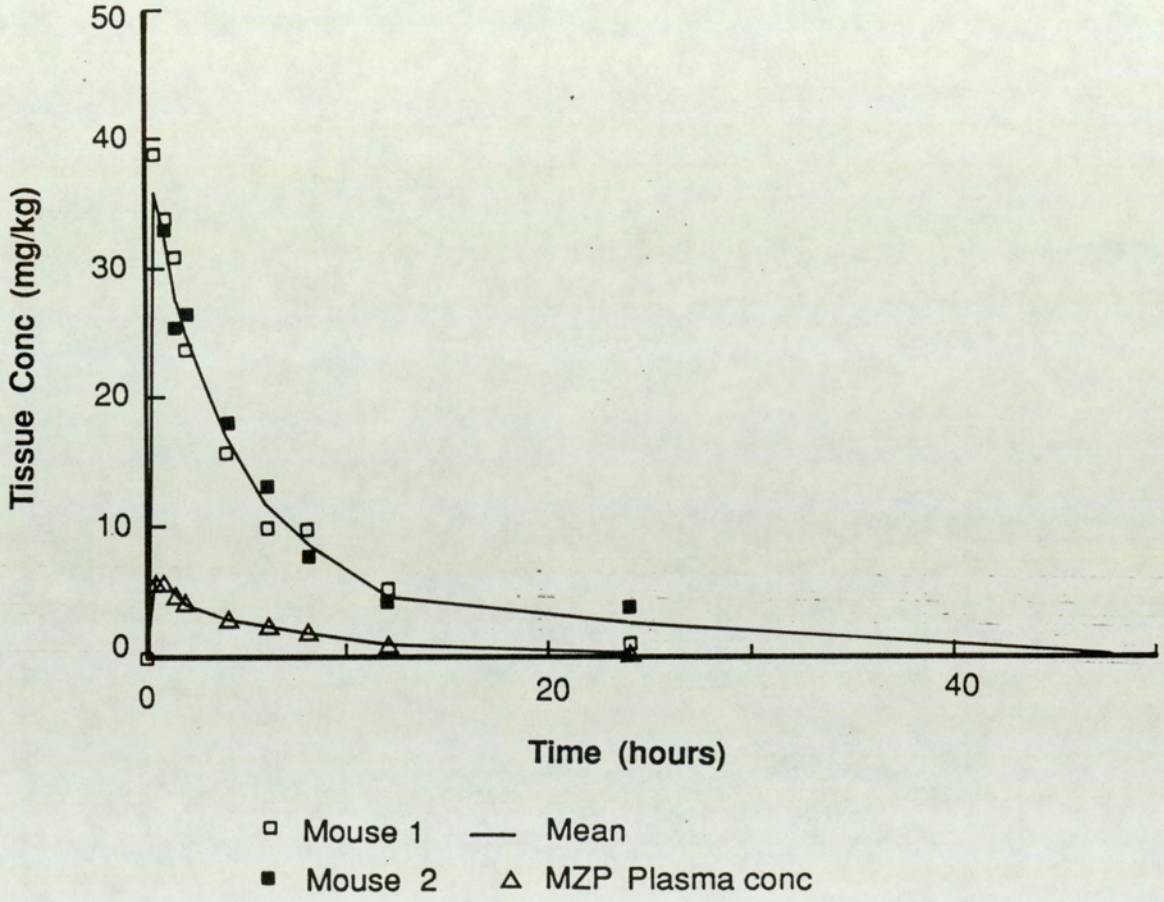
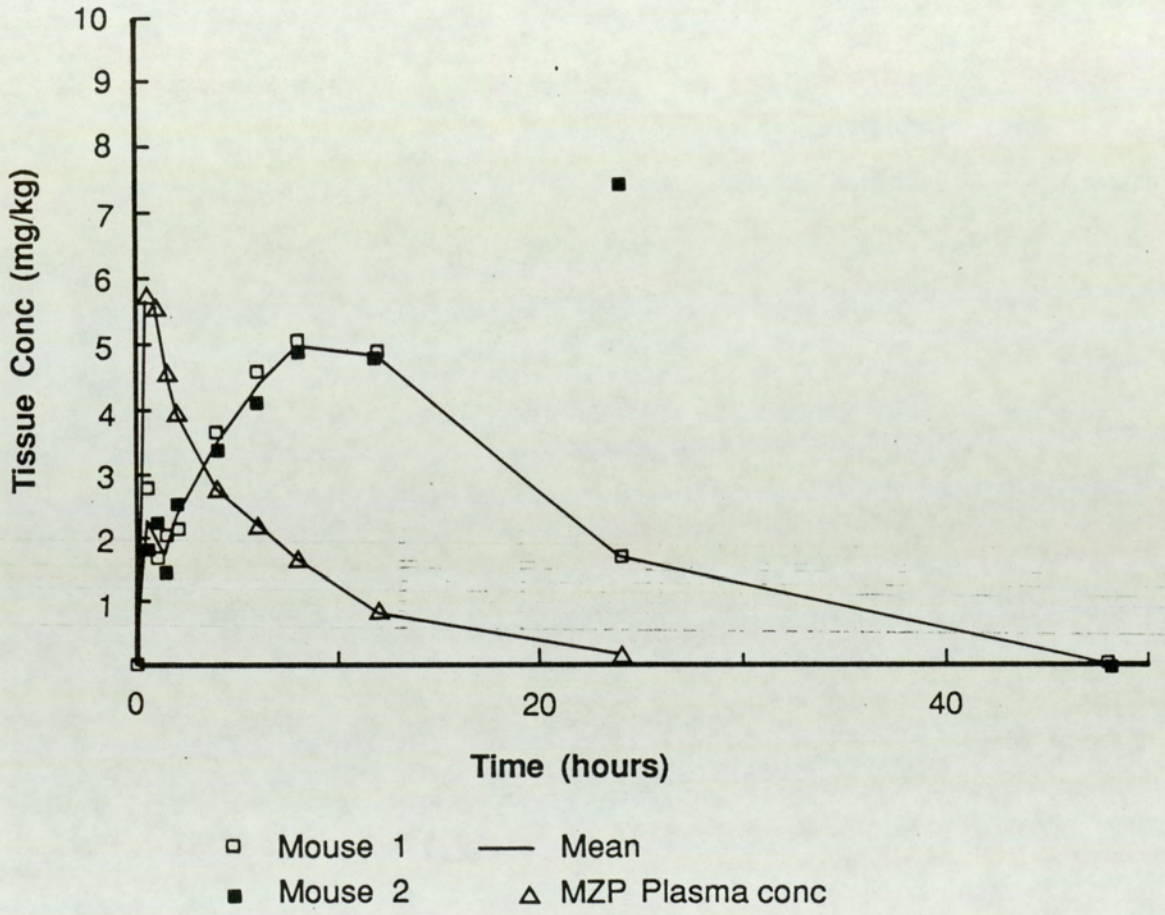


Figure 3.16 MAP brain and MZP plasma concentrations versus time in mice dosed with MZP at 20 mg/kg IP



The concentration of MAP found in the brain of mouse 2 at 24 hours was unexpectedly high thus the line passes through the concentration point for mouse 1 only

Table 3.13 Distribution and apparent elimination half-lives of MZP in some tissues of the mouse after IP administration at 20 mg/kg

Tissue	T_{max} (hr)	C_{max} (mg/kg)	Tissue/plasma concentration ratios	Apparent elimination $t_{\frac{1}{2}}$ (hr)
Liver	0.5	38.43	6.7	3.45
Brain	0.5	36.10	6.3	5.82
Lung	1.5	64.48	14.2	5.76
Heart	0.5	21.90	3.8	5.48
Kidney	0.5	38.70	6.7	6.02

MAP against time and the assay results for both mice at each time-point are shown. Plasma levels of MZP after IP dosage at 20 mg/kg are also presented on the graphs. A detailed breakdown of levels of MZP and MAP found in all tissues examined are tabulated in Appendix 3.

Maximal levels of MZP, the times at which they were reached (T_{max}) and the corresponding tissue/plasma concentration ratios are summarized in Table 3.13. Intact MZP appeared in all the tissues examined 30 minutes after injection. In liver, brain, heart and kidney the highest levels were attained at 30 minutes whereas in lung the maximal level was reached 1.5 hours after injection. The most striking feature of the data in Table 3.13 is that MZP can attain concentrations in tissues threefold higher than the dosage level. Tissue/plasma concentration ratios ranged from 3.8 to 14.2. MZP rapidly crossed the blood-brain barrier of the mouse to reach a concentration in the brain 6.3-fold higher than in the plasma. Within the tissues examined the highest concentration of MZP (64.48 mg/kg) was found in lung. Measureable levels of MZP were sustained by the liver for 24 hours and by the brain, lung, heart and kidney for 48 hours. The apparent

half-lives for elimination of MZP from tissues (Table 3.13) are minimally 3.45 hours (liver) and maximally 6.02 hours (kidney).

MAP was detected in all tissues 30 minutes after injection of MZP. Maximal levels were reached in the liver (28.83 mg/kg) at 1 hour, in the heart (9.28 mg/kg) and kidney (21.55 mg/kg) at 1.5 hours and in the brain (4.98 mg/kg) and lung (18.90 mg/kg) at 8 hours. Measurable levels of MAP were sustained by all tissues examined for 24 hours and for 48 hours by the lung, heart and kidney.

3.10.4 Discussion

MZP was rapidly absorbed into the blood following IP injection and murine plasma concentration-time data could be fitted to a 2-compartmental model. The drug passed quickly into the peripheral compartment, with a distribution half-life of approximately 0.6 hours. After IP injection plasma elimination half-lives were 3.04 hours (10 mg/kg dosage) and 4.58 hours (20 mg/kg dosage). Plasma decay data for some other lipophilic antifolates are presented in Table 1.5. MZP was eliminated from mouse plasma more rapidly than metoprine ($t_{\frac{1}{2}}$ 19 hours) etoprine ($t_{\frac{1}{2}}$ 14 hours) and pyrimethamine ($t_{\frac{1}{2}}$ 6.5 hours) but more slowly than trimetrexate ($t_{\frac{1}{2}}$ 0.83 hours). The total body water of a mouse is 15-18 ml (190) and the apparent V_d of MZP in the mouse (101 ml at 10 mg/kg and 87 ml at 20 mg/kg dosage) demonstrate that tissue binding was extensive or that MZP was widely distributed.

MZP was absorbed somewhat more slowly from the gastro-intestinal tract than from the IP site; following oral administration at 10 mg/kg the Cp_{max} (2.07 mg/L) was reached after 1 hour whereas after IP administration at the same dosage level the Cp_{max} (2.60 mg/L) was attained after 15 minutes. The plasma concentration-time profile after oral dosage was however similar to that seen after IP administration. Oral bioavailability, relative to IP administration, was 1.23, which indicated that absorption

from the gastrointestinal tract was complete and that first-pass hepatic metabolism was negligible.

MZP rapidly entered the liver, brain, lung, heart and kidney of mice after IP administration. The high tissue levels of MZP found in this study are in agreement with reports on the tisse pharmacokinetics of other lipophilic antifolates. In the rat metoprine attained a brain/plasma concentration ratio of 6.5 and a lung/plasma concentration ratio of 30.8 (6,75). In man the highest concentration of metoprine was found in lung and substantial amounts were found in the liver and brain (17,75). Pyrimethamine localized in soft, well-perfused tissues (heart, lung, kidney, liver and muscle) of the mouse, rat and pig (156,158,166,174). DAMP attained high tissue/plasma concentration ratios in the liver (5.8), brain (10.9) and kidney (12.9) of rats (19). BW 301U reached a lung/plasma concentration ratio of 3.5 and a liver/plasma concentration ratio of 7.0 in the rat (77). High levels of trimextrexate were found in the liver and kidney of dogs (128). BW 301U and trimetrexate did not readily penetrate into the CNS however and trimetrexate reached only low levels in lung tissue (77,128). It is the high lipophilicity of the small-molecule antifolates which enables them to distribute widely into different tissues (6).

Substantial levels of MAP rapidly appeared in all the mouse tissues examined after IP administration of MZP at 20 mg/kg. Conversion of MZP to MAP occurred readily in excised mouse liver (Section 3.8) and the results of analysis of both MZP and MAP in liver in the murine pharmacokinetic study must be viewed with some caution. The extent to which biotransformation of MZP to MAP occurred during storage of samples (for 3 weeks) and during the analytical procedure is uncertain. The maximal level of MAP was reached firstly in liver (at 1 hour), then in heart and kidney (at 1.5 hours) and finally in brain and lung (at 8 hours). The MZP concentration fell most rapidly in the liver (elimination $t_{\frac{1}{2}}$ 3.45 hours). These observations suggest that MAP was formed principally in the liver.

The rat excretion balance study (Section 3.9) and the mouse tissue pharmacokinetic work demonstrated that a primary route of metabolism of MZP was conversion to MAP. The amine was not however detected in the plasma of mice at dosages up to 20 mg/kg (IP). It is likely that MAP was present in mouse plasma after administration of MZP, but the levels attained were below the limit of sensitivity of the assay procedure (0.05 mg/L).

3.11 Clinical pharmacokinetics of MZP

The IV dose escalation study aimed at establishing the maximum tolerated dose of the drug in man. The clinical pharmacokinetics of MZP were studied on patients entered into the phase I trial. The analytical procedure was insufficiently sensitive to allow measurement of MZP in the plasma of patients treated with the 3 lowest doses of MZPES (5.4, 11.0 and 18.0 mg/m²) thus the pharmacokinetic study commenced at a dose of 27 mg/m² and progressed through 15 intermediate dose levels to 460 mg/m².

MZP plasma levels were monitored in 38 patients who received 46 doses of MZPES, 23 patients and 30 doses of which were studied in the Birmingham arm of the trial. Blood was sampled in patient 4 for up to 6 hours, patient 2 for up to 8 hours and patients 1 and 3 for up to 12 hours after the start of infusion at 27 mg/m². After the general form of the plasma concentration-time curve had been determined plasma was sampled for up to 48 hours in subsequent studies, but logistic problems prevented samples being taken from patients 5 (38 mg/m²), 10 (67 mg/m²), 16 (83 mg/m²), 22 (150 mg/m²), 24 (177 mg/m²), 25, 27 (200 mg/m²) and 34 (300 mg/m²) beyond 24 hours. The author analysed plasma from 28 of the 30 Birmingham studies. Table 3.14 summarises the number of patients and courses of treatment evaluated at each dose level and shows the distribution of these between the 2 study centres. Doses of 200, 210 and 215 mg/m² were viewed

Table 3.14 The number of patients and courses evaluated at each dose in the phase I trial

Dose (mg/m^2)	27	38	50	67	83	105	125	150	177	207	250	300	360	400	460
No. of patients															
Birmingham	3	0	1	3	4	3	1	2	0	3	2	0	2	1	1
Charing Cross	1	1	2	2	1	1	0	0	1	4	0	2	1	0	0
TOTAL	4	1	3	5	5	4	1	2	1	7	2	2	3	1	1
No. of courses															
Birmingham	4	0	1	3	6	3	1	2	0	4	2	0	2	1	1
Charing Cross	1	1	2	2	1	1	0	0	1	4	0	2	1	0	0
TOTAL	5	1	3	5	7	4	1	2	1	8	2	2	3	1	1

as a single dose level (207 mg/m²) in this table and in all subsequent pharmacokinetic calculations.

In individual studies at Birmingham 23 to 177 days elapsed between collection of samples and analysis. Initially 4 quality control plasma samples were included in each patient-dose series but this was reduced to 2 per series as confidence in the analytical procedure was established. Analytical results from a series of test plasmas were accepted if assays of quality control samples lay within the range ± 15% of predetermined values.

Only MZP was detected in the plasma of the majority of patients. Very low levels of a material corresponding in retention time to MAP were seen in 24 and 48 hour samples taken from patient 32 treated at 250 mg/m² and patients 36 and 37 treated at 360 mg/m². MAP-Ac was not detected in the plasma of any patient at any time-point. Chromatograms of pre-dose samples in 3 courses showed peaks corresponding closely to MZP and pyrimethamine, thus the analytical results from these studies were omitted from the pharmacokinetic evaluation. Patients entered into a phase I trial have often received prior chemotherapy and it is probable that the interfering peaks were due to other drugs.

Pharmacokinetic profiles for 3 courses of treatment are presented in Figures 3.17, 3.18 and 3.19. Mean measured plasma concentrations (mg/L) of MZP are shown on the graphs and the data is regressed to produce fitted lines. The post-infusion plasma concentration time profiles are biphasic, which suggested that MZP followed 2-compartment kinetics in man.

The mean plasma levels of MZP determined by the author, together with Ln concentration and fitted concentrations (arithmetic and Ln) are presented in Appendix 4. A detailed synopsis of the pharmacokinetic parameters evaluated for all patients in the clinical trial (both Birmingham and Charing Cross) is presented in Appendix 5. Table 3.15 summarizes the mean pharmacokinetic

Figure 3.17 MZP plasma pharmacokinetics in man. Patient 12 administered MZPES at 67 mg/m^2

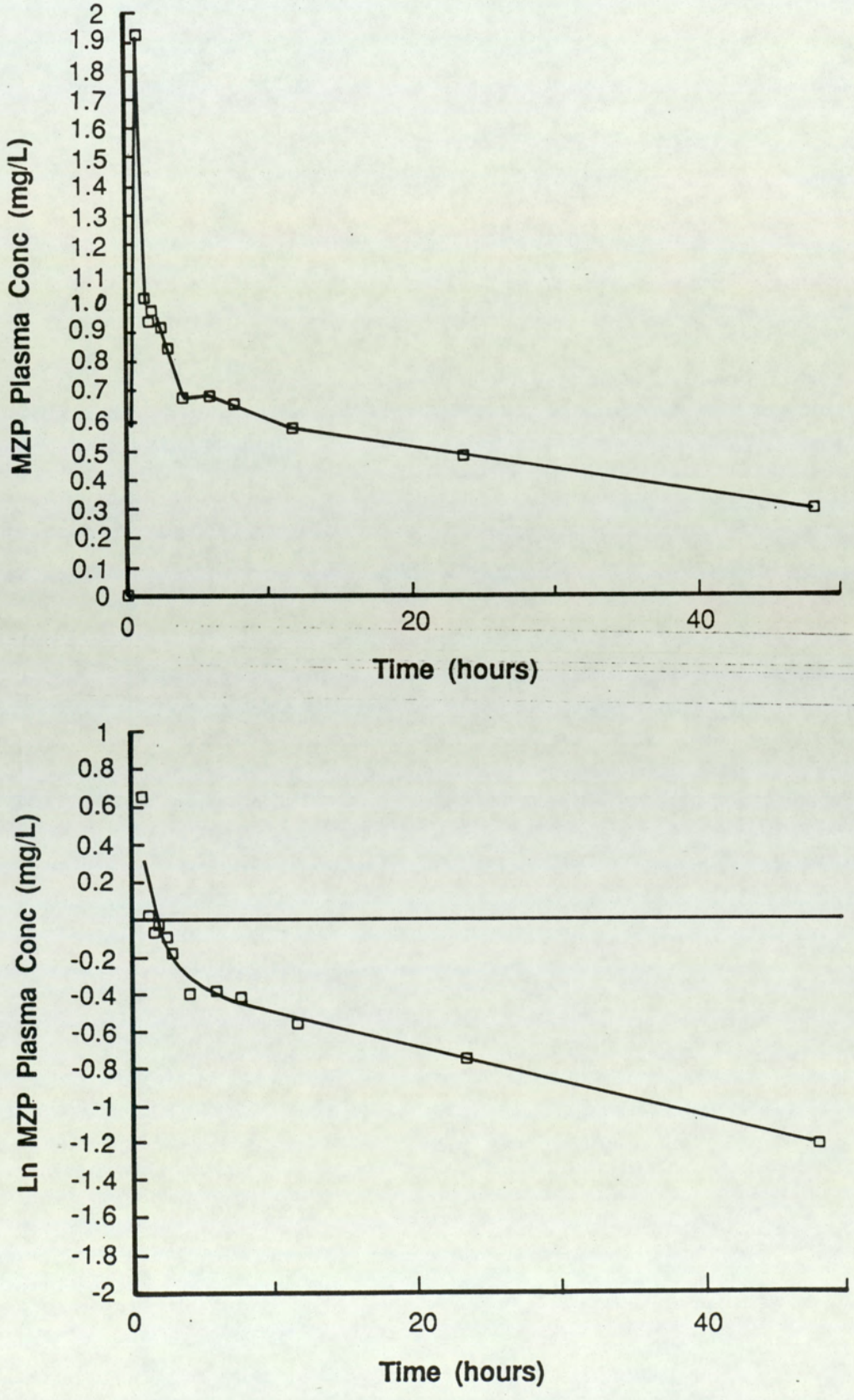


Figure 3.18 MZP plasma pharmacokinetics in man.
Patient 37 administered MZPES at 360 mg/m^2

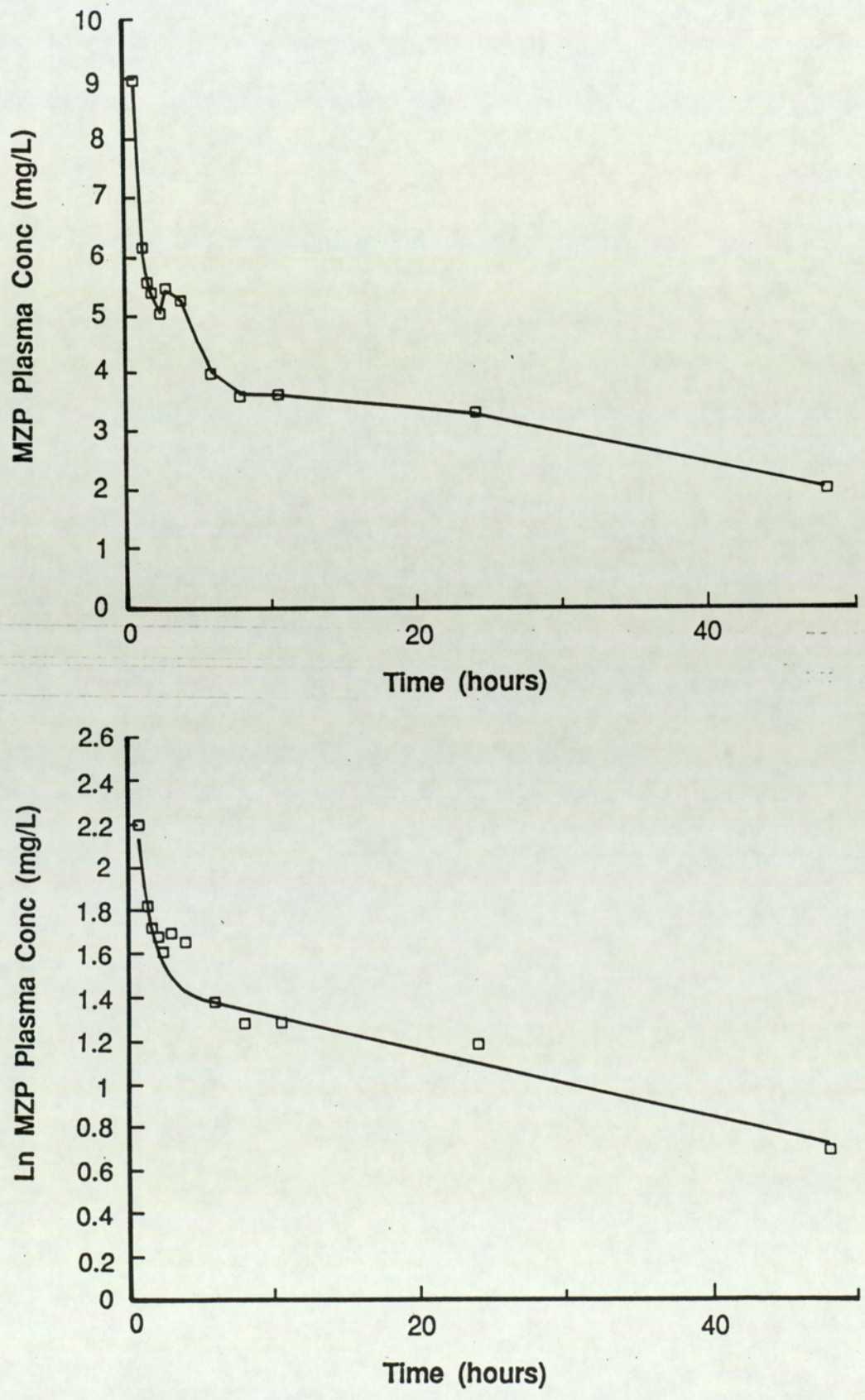
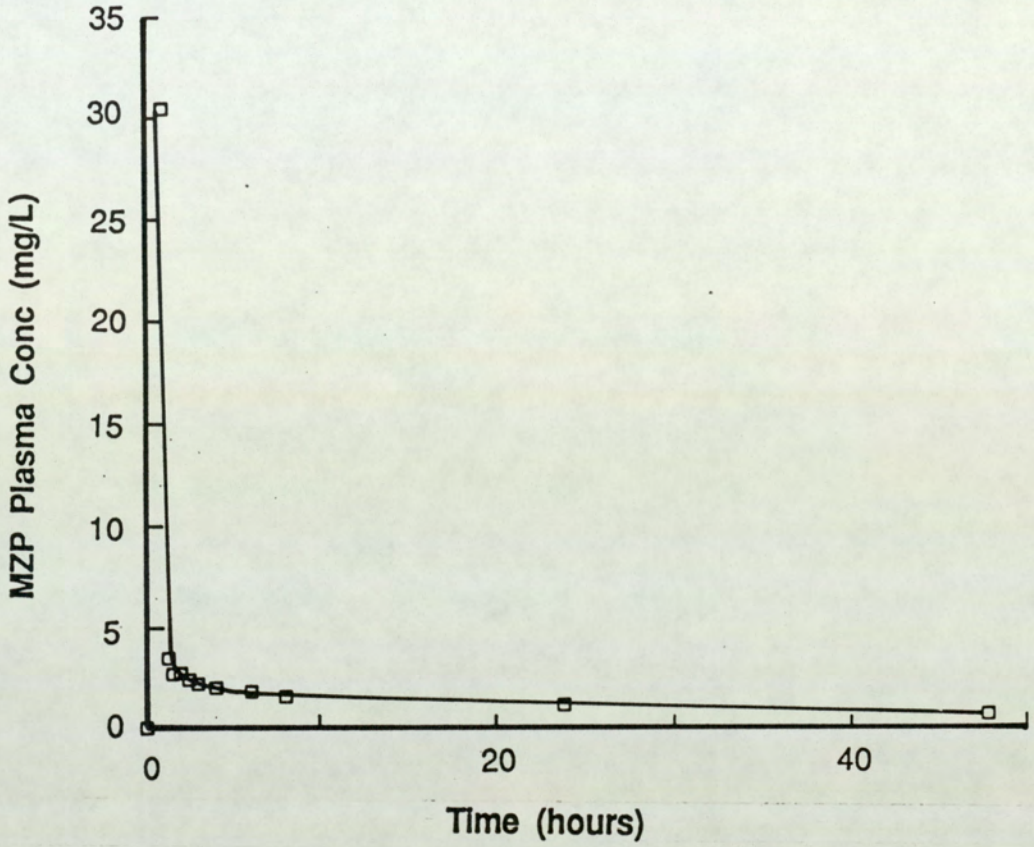


Figure 3.19 MZP plasma pharmacokinetics in man.
Patient 30 administered MZPES at 210 mg/m^2



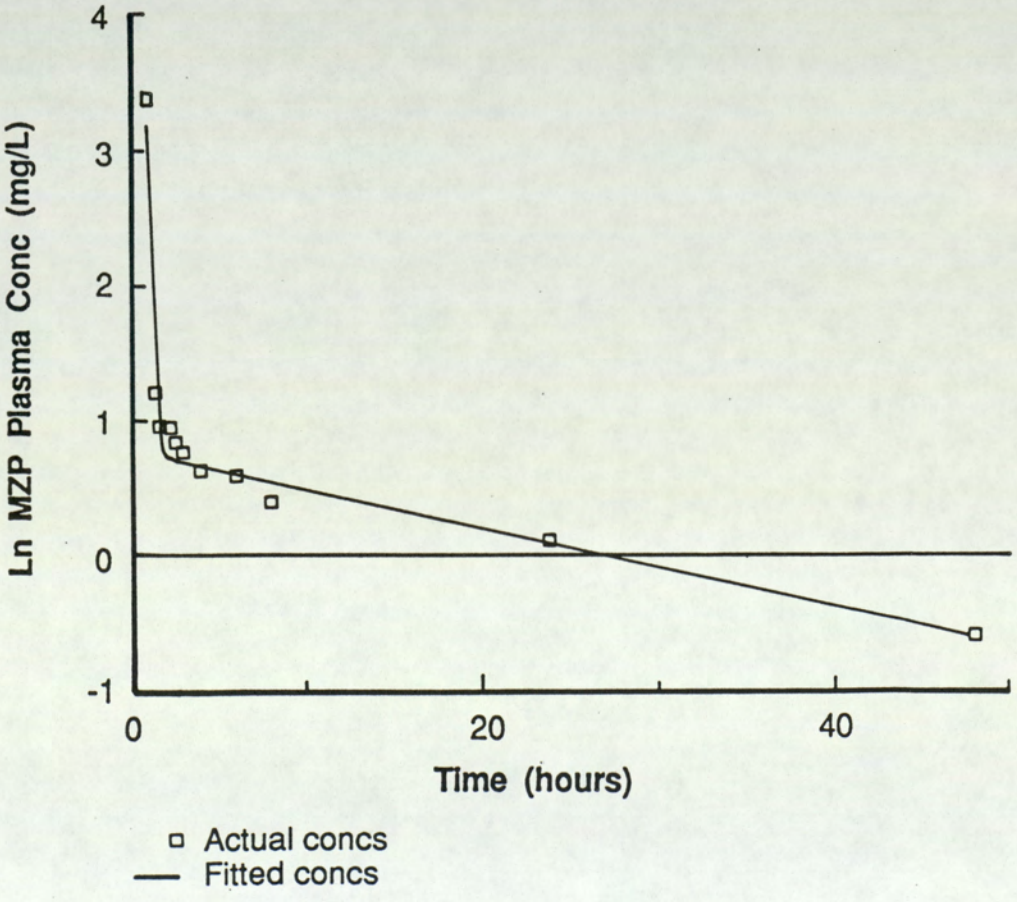


Table 3.15 Mean pharmacokinetic parameters evaluated at each dose level in the human dose escalation study

Dose (mg/m^2)	n		Cp_{max} (mg/L)	AUC_{trap} (mgL/hr)	AUC_{0-inf} (mgL/hr)	Vol central compartment (L)	Apparent Vd (L)	Distribution $t_{\frac{1}{2}}$ (hr)	Elimination $t_{\frac{1}{2}}$ (hr)	Plasma clearance (L/hr)
27	5		0.454	2.382	12.456	-	152.510	0.135	33.167	21.778
		CV(%)	30.0	42.0	68.7	-	49.2	-	72.8	51.4
38	1		1.964	8.444	23.127	-	133.551	-	41.020	8.527
50	3		5.445	13.529	21.816	9.972	198.938	0.640	43.163	5.659
		CV(%)	145.7	12.9	67.8	-	59.7	-	60.6	18.9
67	5		2.071	18.744	27.622	18.690	123.638	0.288	27.360	6.544
		CV(%)	54.1	30.6	13.8	74.7	40.9	88.1	31.6	24.6
83	7		2.407	24.985	40.273	30.327	126.349	0.442	29.640	6.481
		CV(%)	49.8	14.9	21.4	97.9	19.5	150.3	9.7	11.3
105	4		2.081	33.762	61.134	21.384	123.825	0.192	38.345	5.693
		CV(%)	28.7	25.5	45.4	90.4	19.5	76.2	34.1	29.6
125	1		1.858	36.731	101.824	19.642	199.924	0.101	76.670	6.915
150	2		6.219	43.951	71.878	20.141	92.782	0.214	23.133	6.115
177	1		4.099	45.827	65.328	8.905	55.031	0.049	13.300	5.630

207	8		7.704	66.899	107.516	36.115	90.579	0.181	26.798	5.611
		CV(%)	123.4	26.5	19.2	86.3	18.0	99.8	25.0	32.1
250	2		12.594	107.940	169.138	18.141	78.920	0.147	31.007	3.963
300	2		6.908	111.935	438.083	42.909	95.501	1.538	90.680	4.308
360	3		7.267	127.150	175.073	26.417	94.850	0.224	25.762	4.925
		CV(%)	22.1	26.9	33.5	76.7	29.7	67.7	21.3	33.5
400	1		10.221	126.960	264.946	31.647	61.181	0.399	26.269	4.624
460	1		8.888	164.843	213.090	48.794	93.465	0.267	22.124	5.217

- calculated as single compartment model.

Table 3.16 Overall mean values for pharmacokinetic parameters in the human dose escalation study (46 courses of treatment)

	Vol central comparment (L)	V_d (L)	Distribution $t_{\frac{1}{2}}$ (hr)	Elimination $t_{\frac{1}{2}}$ (hr)	Plasma clearance (L/hr)
	27.309	118.349	0.267	33.775	7.523
CV(%)	84.6	44.6	182.7	60.7	82.3

parameters evaluated at each dose level, together with CVs for these values and Table 3.16 presents overall mean values for central compartment volume, V_d, distribution half-life, elimination half-life and plasma clearance with their respective CVs.

All pharmacokinetic parameters showed high inter-patient variability, even at individual dosage levels. For example, the CV for Cp_{max} ranged from 22% at 360 mg/m² (n=3) to 146% at 50 mg/m² (n=3) and the CV for plasma elimination half-life was minimally 10% at 83 mg/m² (n=7) and maximally 73% at 27 mg/m² (n=5).

Figure 3.20 is a computer-generated plot of mean AUC_{trap} at each dose level against ascending dose. The correlation coefficient for the curve is 0.987. The correlation between AUC_{trap} for all courses and ascending dose was rather less satisfactory (r = 0.954). Correlations of dose to mean AUC_{0-inf} at each dose level and to AUC_{0-inf} for all courses were modest (r = 0.779 and 0.762 respectively).

Plasma drug levels fell considerably in the first hour after infusion. Figure 3.21 is a computer-generated graph of mean Cp_{max} at each dose level versus dose and the correlation coefficient for the plot is 0.778. The correlation between Cp_{max} for all courses and dose was poor (r = 0.474).

Figure 3.20 MZPES phase 1 trial. Mean trapezoidal AUC (MZP) at each dose level versus dose

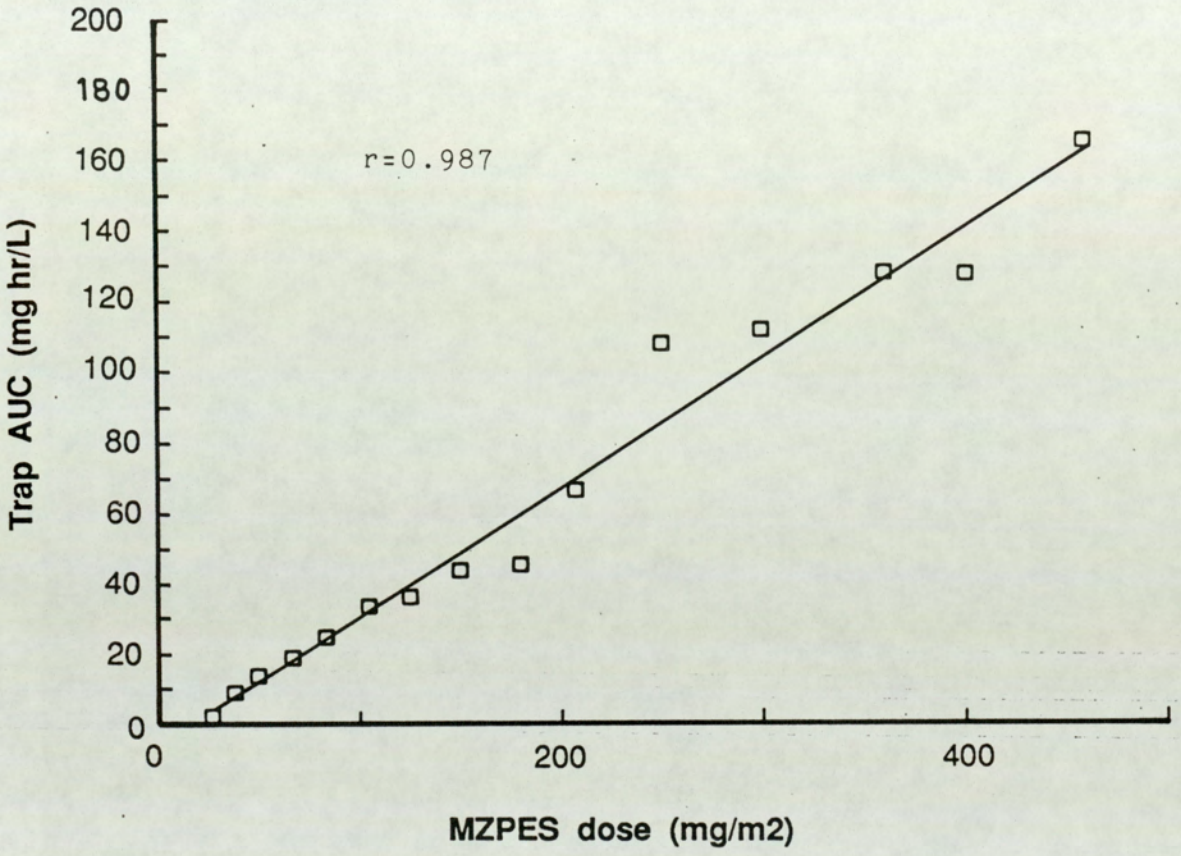
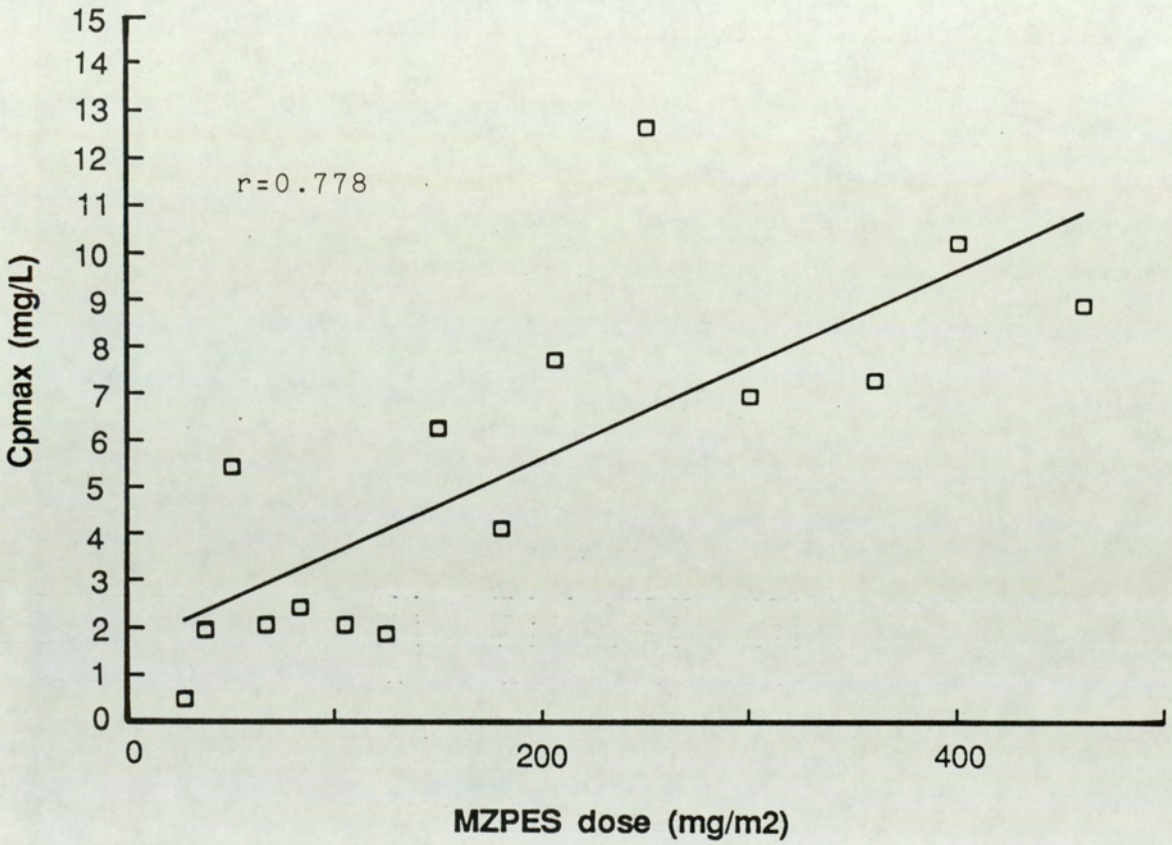


Figure 3.21 MZPES phase 1 trial. Mean maximal plasma concentration (MZP) at each dose level versus dose



Calculated values for the central compartment volume and the V_d are presented in Tables 3.15 and 3.16 and Appendix 5. Over the 46 doses administered the mean central compartment volume was 27L (CV 85%). In individual patients the calculated values were minimally 0.808 L (patient 30 treated at 210 mg/m²) and maximally 90.365 L (patient 12 treated at 83 mg/m²). The mean V_d was 118 L (CV 45%). Apparent V_d values ranged from 61 L (patient 37 dosed at 400 mg/m²) to 332 L (patient 7 dosed at 50 mg/m²).

Plasma half-life and clearance data for MZP in man is presented in Tables 3.15 and 3.16 and Appendix 5. The overall mean distribution half-life was 0.27 hours (CV 183%). Individual distribution half-lives were minimally 0.04 hours (patient 29 dosed at 210 mg/m²) and maximally 2.79 hours (patient 33 treated at 300 mg/m²). The overall mean elimination half-life was 34 hours (CV 61%) and individual values ranged from 10.39 hours (patient 4 dosed at 27 mg/m²) to 131.86 hours (patient 33 treated at 300 mg/m²). The overall mean plasma clearance was 7.5 L/hr (CV 82%). Individual values were minimally 3.04 L/hr (patient 32 treated at 250 mg/m²) and maximally 40.2 L/hr (patient 2 treated at 27 mg/m²).

3.11.1 Discussion

Measurable objective tumour regressions were not observed in any of the patients treated in the phase I clinical trial. The primary aim of the dose escalation study however was to establish the maximum tolerated dose of MZPES and at the highest dose administered (460 mg/m²) nausea and vomiting were experienced by some patients.

Human plasma concentration-time data could be fitted to a 2-compartment model following a 1 hour IV infusion of MZPES. The central compartment volume for MZP was 27 litres. This compartment is usually made up of blood and those organs or tissues which are highly perfused. After equilibrium had been established throughout the central compartment the drug passed rapidly into the

peripheral compartment with a distribution half-life of 0.27 hours. The approximate volumes of the body water compartments for a person of average weight are: intracellular water 25 litres; extracellular water 17 litres (of which 3 litres is plasma water) (170). The apparent V_d of MZP in man (118 L) exceeded the total volume of body water (42 L) by a factor of 2.8, thus the drug was able to distribute beyond total body water and reach poorly perfused areas, or was highly bound in well perfused areas of the body.

The plasma elimination half-life of MZP in man was 34 hours. Thus the drug is removed from human plasma more rapidly than metoprine ($t_{\frac{1}{2}}$ 216 hours), etoprine ($t_{\frac{1}{2}}$ 176 hours) and pyrimethamine ($t_{\frac{1}{2}}$ 85 hours) but more slowly than MTX ($t_{\frac{1}{2}}$ 30 hours), trimetrexate ($t_{\frac{1}{2}}$ 16.4 hours) and BW 301U ($t_{\frac{1}{2}}$ 4.5 hours)(Table 1.5).

There was an approximately linear correlation between mean trapezoidal AUC at each dose level and ascending dose ($r = 0.987$, Figure 3.20). There was no clear-cut relationship between dose level and form of the concentration-time curve, nor was there any apparent relationship between dose and central compartment volume, apparent V_d, and clearance. Correlations between Cp_{max} and dose were poor however, as were the relationships between AUC_{inf} and dose. This implies that in this case calculation of AUC_{trap} is superior to evaluation of AUC_{inf} when attempting to relate AUCs to dosage. The weight of evidence is in favour of no dose-dependency in the pharmacokinetics of MZPES over the dosage range of 27 to 460 mg/m². This suggests that any contribution from metabolism or excretion to the fate of MZP in man is <u>via</u> mechanisms that are not saturable over the dosage range covered. The elimination of drugs is determined by contributions from metabolism, chemical degradation and excretion. Metabolism and excretion are non-reversible processes and the elimination rate constant is effectively the sum of these 3 contributing mechanisms.

In the Birmingham arm of the trial the time between

sample collection and analysis was rather protracted (23 to 177 days). However, plasma from patients in the study showed maximally 10.9% loss of drug after storage at -20°C in Sterilin vials for 455 days (Table 3.11). The results of the human pharmacokinetic study would therefore appear to be valid within the limits of accuracy of the analytical method.

The importance of the calculated pharmacokinetic parameters with respect to the therapeutic potential of MZP is discussed in Section 4.

Figure 3.17 is a typical plasma pharmacokinetic profile for a patient (no. 12) administered a low dose of MZPES (67 mg/m²). The patient was rather obese (weight 90.72 kg, body surface area 2.044 m²). The infusion time was 0.98 hour and plasma was analysed 46 days after sampling. Cp_{max} (1.925 mg/L) and AUC_{trap} (24.387 mg L/hr) values were close to the means for the dosage group. The central compartment volume (17.369 L), V_d (109.279 L), distribution half-life (0.164 hr), elimination half-life (24.36 hr) and clearance (5.618 L/hr) compared favourably with the overall means for these values. A representative plasma concentration-time curve for a patient (no. 37) given a high dose of MZPES (360 mg/m²) is presented in Figure 3.18. She was below average height and underweight (height 1.600 m, weight 47.9 kg, body surface area 1.474 m²). MZPES was infused over 0.83 hour and samples were analysed 76 days after collection. Cp_{max} (8.976 mg/L) and AUC_{trap} (157.062 mg L/hr) were close to the dosage group mean values and the central compartment value (28.030 L), V_d (74.686 L), distribution half-life (0.329 hr), elimination half-life (31.836 hr) and clearance (3.381 L/hr) did not diverge appreciably from the mean values, within the overall variability of calculated values experienced in this study.

Many of the human plasma concentration time profiles for MZP showed an inflexion 2 to 7 hours after the start of the infusion. This is evident in Figures 3.17 and 3.18 and may be indicative of biliary recycling.

An atypical plasma curve was shown by patient 30 administered MZPES at 210 mg/m² over 0.92 hour (Figure 3.19). The patient was of normal size. The Cp_{max} for MZP was very high (30.567 mg/L, mean for dosage group 7.704 mg/L) and the calculated central compartment volume was very low (0.808 L, overall mean 27.309 L). The distribution half-life (0.084 hr), elimination half-life (18.269 hr) and V_d (72.316 L) were also low in comparison with the overall mean values.

Infusion times for the MZPES - dextrose formulation ranged from 0.45 hours to 1.75 hours in the study. Patient 16, administered MZPES at 83 mg/m² over 0.45 hour, exhibited a very short distribution half-life (0.075 hr). Patient 33 received an infusion at 300 mg/m² over 1.75 hours and demonstrated the longest distribution half-life (2.785 hr) and elimination half-life (131.856 hr) seen in the entire study.

4 GENERAL DISCUSSION

Despite the usefulness of MTX in the treatment of a variety of neoplastic diseases the natural and acquired resistance of tumours to the drug (2,4,5,6) impose limitations on its clinical effectiveness. Laboratory and clinical experience have shown that the activities of the antitumour antifolates are widely diverse. MZP is a non-classical DHFR inhibitor which is not a structural analogue of MTX or dihydrofolic acid. Its broad spectrum of activity against malignant cells in murine models and its cytotoxic effect against transport-deficient MTX-refractory cell lines (Sections 1.6 and 1.7) offer the possibility of clinical value that might be dependent on significant biochemical differences between normal and neoplastic cells.

The lipophilic antifolates enter cells rapidly by a route different to that used by MTX (7-10,77,79) but cell membrane transport may be by a process more complex than passive diffusion (99). The aromatic azido substituent in MZP imparts additional lipophilicity to the pyrimethamine molecule (Table 1.1) and should facilitate cellular uptake and subsequent association with the target enzyme. MZP is 39% ionized at plasma pH (Table 1.1) thus adequate concentrations of neutral base (for cell penetration) and N-1 protonated species (for enzyme inhibition, Section 1.5) should be available in vivo.

MZP is a potent inhibitor of DHFR. Inhibition of this enzyme leads to disruption of nucleic acid synthesis and cell death (Section 1.5). Facile cell entry may circumvent resistance caused by generation of elevated levels of DHFR. In the search for new antitumour antifolates it seems appropriate to study in a systematic way the interaction of drugs with DHFR from MTX-sensitive and MTX-resistant mammalian cells, including samples from patients, in order to uncover possible subtle but exploitable differences in the susceptibilities of enzymes from various types of neoplastic cells (33). There is, however

little definitive evidence to show that the metabolic pathways and the structure of the enzyme itself are different in the tumour cell and the normal cell, thus the targetting of this enzyme has many drawbacks. Trimetrexate is effective against some opportunistic protozoa which frequently infect AIDS patients (191,192,193) and the favourable cell uptake properties of MZP and its efficacy as an inhibitor of DHFR may enable the azide to have similar activity. A desirable characteristic of "second generation" antifolates is potent inhibitory activity not only for DHFR but also for thymidylate synthase (194) and Dolnick and Cheng (94) demonstrated that DAMP displayed some activity against this enzyme. MZP may have sites of cytocidal action supplementary to DHFR, as do metoprine, DAMP and trimetrexate (86,90-93).

The mouse tissue distribution study (Section 3.10) showed that MZP was highly localized from plasma into tissues. Its high affinity for tissues was emphasized by V_ds exceeding total body water 5.7 fold in the mouse (Section 3.10) and 2.8-fold in man (Section 3.11). Thus MZP is able to reach poorly perfused areas of the body, such as adipose tissue, or is highly bound in well perfused regions. Amongst the various mouse tissues analysed the highest concentration of MZP was found in lung. The observations that metoprine (6,75) and MZP show high affinity for lung tissue may indicate a use of these drugs for treatment of lung cancer. MZP rapidly crossed the blood-brain barrier of the mouse to reach a concentration in the brain 6-fold higher than in plasma. The ability of MZP to enter the CNS may permit activity against meningeal cancers and CNS leukaemia. Normal brain tissue may lack DHFR (6,35) and, if so, the 2,4-diaminopyrimidine antifolates will have some selective action on CNS neoplasms. Since trimetrexate reached only low levels in lung tissue and trimetrexate and BW 301U did not readily penetrate into the CNS (77,128) MZP may possess an advantage over those drugs inasmuch as there is a potential for treatment of neoplasms in these areas.

It is the high lipophilicity of the low molecular weight antifolates which enables them to penetrate cell membranes easily (7-9,77,79) and to distribute widely and rapidly into different body compartments (6). However, Selassie et al (80) have observed that, although the advantages of lipophilicity are evident, one must be able to separate the contributions of electronic and steric factors from lipophilicity when discussing drug action. No systematic effort has been made to define what is the upper limit to the beneficial effects of lipophilicity and one cannot expect the effectivness of drugs to increase linearly with lipophilicity (or any other property) indefinitely.

The mouse liver incubation experiment (Section 3.8), the rat excretion study (Section 3.9) and the murine tissue investigation (Section 3.10) demonstrated that a primary route of metabolism of MZP was conversion to MAP. Amongst the mouse tissues examined MZP levels fell most rapidly in the liver, which suggested that this organ was the principal site of biotransformation. Substantial levels of MAP were detected in brain, lung, heart and kidney as well as in liver. Reductive degradation of MZP to MAP introduces an additional basic site into the molecule to afford a more polar compound which should not readily enter tissues. This would suggest that MAP was formed in brain, lung, heart and kidney and the contribution to levels of the metabolite in these tissues by transport from the liver was minimal. It would be possible to test this hypothesis by performing further incubation experiments and determining whether the other tissues have any capacity to convert MZP to MAP. The amine is not apparently produced by reaction of MZP with the cellular thiols reduced glutathione or L-cysteine (Section 3.7) and an incubation experiment using pre-heated liver would demonstrate whether or not the conversion was mediated by an enzyme. Plasma and urine did not contain any agent which could reduce MZP to MAP (Section 3.6). Since MAP is more polar than MZP it should be more

rapidly excreted than the parent and in the rat MAP elimination exceeded that of MZP. Because MAP appeared in the urine and faeces of MZP-treated rats it is likely that the metabolite was present in the plasma of mice (Section 3.10) and patients (Section 3.11) after administration of the azide. MAP was not however detected in the plasma of mice after dosage with MZP up to 20 mg/kg (IP) and only very low levels of material corresponding in retention time to the amine were seen in 24 and 48 hour plasma samples taken from patients administered MZPES at 250 mg/m^2 (1 patient) and at 360 mg/m^2 (2 patients). The plasma concentrations of MAP attained in mice and men were probably below the sensitivity limit of the assay procedure.

Some kidney and heart extracts from MZP-treated mice (Section 3.10) showed very low levels of a material corresponding in retention time to MAP-Ac. Low concentrations of the amide were detected, albeit without certainty, in the urine and faeces of MZP-treated rats (Section 3.9). The poor solubility of MAP-Ac in plasma and urine (Section 3.6) may tend to detract from formation of the amide as a means of eliminating the foreign compound (MZP) from the body. The azido function may inhibit metabolic acetylation and this proposition could be tested by dosing animals with a drug known to be readily acetylated and co-administering an azide.

MZP, MAP and MAP-Ac were tentatively identified in urine, faeces and liver of test animals by measurement of ultraviolet absorbance ratios (Section 3.4). Greater confidence in identification would be provided by collection of appropriate chromatographic fractions and examination of the NMR and mass spectra of isolated compounds.

In the rat excretion balance study (Section 3.9) the maximum overall recovery of MZP and MAP in urine and faeces during 72 hours was 14%. The analytical method employed throughout the present work was capable of detecting only unconjugated MZP, MAP and MAP-Ac but it is likely

that MZP is converted in vivo to a wide variety of metabolites (Section 1.10.4). Probable metabolic routes (supplementary to formation of MAP and MAP-Ac) are: hydroxylation of the 6-ethyl side chain at the alpha-position; N-1 oxidation; N-3 oxidation (more extensive than N-1 oxidation because of steric hindrance from the 6-ethyl, or alpha-hydroxylated 6-ethyl, group); conjugation of MZP or its phase I metabolites with glucuronic acid. Several of these reactions may occur within individual molecules, for instance the formation of MAP-N-3-oxide, or 6-ethyl alpha-hydroxylated MAP-Ac, can be envisaged. If a metabolic pathway can be saturated this may determine the degree of biotransformation by the alternative routes.

For a drug with one or more active metabolites a rigorous correlation of drug plasma level with pharmacological response is impossible without a detailed knowledge of both the relative potencies and the pharmacokinetic characteristics of the individual active metabolites (24). MAP was 50-fold less active than MZP as a DHFR inhibitor (88). Development of more selective and effective DHFR inhibitors may involve exploitation of cellular differences such as drug transport and metabolism rather than enzyme differences (83).

The TLC studies (Section 3.2) did not contribute to knowledge of the pharmacokinetics or metabolism of MZP but may be of use in disposition studies using radiolabelled MZP. Methods of analysis for the N-oxides and glucuronide conjugates of the drug are required. Drug conjugates and N-oxides are very polar and not easily extracted from aqueous systems. Conjugates can be hydrolyzed by either acidic or enzymic methods and N-oxides can be reduced. In both cases either the parent drug or primary metabolites will be re-formed which can subsequently be extracted to measure the amount of polar metabolite present (195).

MZP, MAP and MAP-Ac were stable in spiked plasma and urine for at least 11 hours at 37°C (Section 3.6) thus chemical change did not appreciably contribute to

pharmacokinetic parameters for these compounds measured *in vivo*. In the mouse the oral bioavailability of MZP, relative to IP administration, exceeded 95% (Section 3.10) thus absorption from the gastrointestinal tract was complete and presystemic metabolism in the intestinal mucosa or first-pass biotransformation in the liver was negligble.

To produce a drug with a short half-life a 6-ethyl group (as in MZP, pyrimethamine and etoprine) is preferable to a 6-methyl group (as in metoprine) because the latter group does not apparently undergo biotransformation in 2,4-diaminopyrimidines (Section 1.10.4). MZP was eliminated from mouse plasma ($t_{\frac{1}{2}}$ about 3.8 hours, Section 3.10) and human plasma ($t_{\frac{1}{2}}$ 34 hours, Section 3.11) more rapidly than pyrimethamine ($t_{\frac{1}{2}}$s 6.5 and 85 hours respectively, Table 1.5). Thus introduction of an azido group into pyrimethamine results in a molecule with a shorter biological half-life. MZP closely resembles etoprine in structure and the lipophilicity of the azide approaches that of etoprine (log P 2.96 and 3.19 respectively, Table 1.1). The plasma half-lives of MZP in mouse and man were considerably shorter than those of etoprine (14 and 176 hours respectively, Table 1.5) thus replacement of the 3'-chloro group in etoprine by a 3'-azido group produces a molecule which is removed from the plasma more rapidly. MZP, pyrimethamine and etoprine should undergo N-oxidation, 6-ethyl alpha-hydroxylation and phase II conjugation with equal ease thus the short half-life of MZP would appear to be due to rapid metabolism to MAP. This is difficult to reconcile with the apparent absence of the metabolite from the plasma of mice and men treated with MZP however, and the principal metabolite which appears in blood may be N-oxidized, 6-ethyl alpha-hydroxylated or conjugated MAP.

It is probably the protracted biological half-life of metoprine which causes the drug's cumulative toxicity to normal cells (20,22-24,26). A lipophilic antifolate with a shorter half-life, such as MZPES, should display lesser toxicity which could be more readily controlled by dosage scheduling. In the search for novel lipophilic

antifolates a principal aim should be an optimal half-life, which achieves maximal cell kill with minimal toxicity. MZPES was considerably less toxic than metoprine in the mouse (LD_{50} IP 75 and 38 mg/kg respectively, Table 1.3) and in one clinical evaluation of metoprine (24) bone marrow suppression was evident at a dose of 65 mg/m² (PO), whereas in the present study MZPES was administered at doses up to 460 mg/m² (IV) without any evident haematological toxicity. This lack of toxicity of the azide is difficult to explain but may well prove to be of value in future studies on lipophilic antifolates and possibly other drug groups. It is reasonable to predict that at higher doses MZPES will not remain devoid of folate-related toxicities (bone marrow suppression and gastrointestinal effects) but its short half-life should allow design of an optimal dosage schedule of drug and folinic acid so as to provide a safe but effective combination. Haematological and gut toxicities of metoprine were ameliorated by folinic acid only with difficulty because of the long half-life of the antifolate (20,30,31,56,59,60,112).

DAMP was a very effective inhibitor of HMT (28,29) and was acutely neurotoxic (19). These would appear to be the disadvantages that prevented the adamantyl derivatives from entering clinical trial. BW 301U had weak activity as an inhibitor of HMT (28,29). It did not cross the blood-barrier (77) thus was devoid of troublesome CNS toxicity, but it did exhibit antifolate toxicity (116). Phase I clinical trials of both BW 301U (114-116) and trimetrexate (117-121) have been reported. BW 301U has a potential advantage over trimetrexate by virtue of significantly lower risk of histamine-related side effects (28,29). The dose-limiting toxicity of trimetrexate was however haematological and the dose recommended for phase II clinical study was 120 mg/m² every 2 weeks (119).

MZP is the first aromatic azide to enter the clinic (Sections 2.10 and 3.11). The drug is easily formulated as a 1% w/v aqueous solution of the ethanesulphonate salt. All pharmacokinetic parameters evaluated following a 1 hour

IV infusion of the drug showed high inter-patient variability, even at individual dosage levels. This may reflect variable distribution, metabolism or excretion. Over the dose range studied (27 to 460 mg/m²) there was an approximately linear correlation between mean trapezoidal AUC at each dose level and ascending dose ($r = 0.987$, Figure 3.20). The correlation between mean Cp_{max} at each dose level and ascending dose was modest ($r = 0.778$, Figure 3.21). There was no apparent relationship between dose and central compartment volume, V_d, half-lives or clearance. Thus it is unlikely that problems associated with dose-dependency will be encountered in the clinical use of MZPES.

Human plasma concentration-time profiles could be fitted to a 2-compartment model. MZP distributed into an initial volume of 27 litres (0.415 L/kg for a 65 kg man, or 41.5% body weight). After equilibrium had been established throughout the central compartment MZP passed rapidly into the peripheral compartment with a distribution half-life of 0.27 hours. The V_d was 118 litres, or 1.815 L/kg for a 65 kg man. The corresponding value for trimetrexate was 0.62 L/kg (119). MZP is considerably more lipophilic than trimetrexate (log P values 2.96 and 0.88 respectively, Table 1.1) and this may be one factor related to the more extensive tissue distribution of the azide.

The mean plasma elimination of MZP in man was 34 hours. Corresponding values for other antitumour antifolates are: metoprine 216 hours, MTX 30 hours; trimetrexate 16.4 hours; BW 301U 4.5 hours (Table 1.5). Thus in order to achieve adequate intracellular concentrations for the length of time required for cell kill MZP would require more frequent administration, or infusion over a longer period, than that required for metoprine, but less frequent administration, or infusion over a shorter period, than that required for MTX, trimetrexate or BW 301U.

The plasma clearance of MZP was 7.5 L/hr. The corresponding value for trimetrexate was 3.2 L/hr (119) and

the total body clearance of BW 301U was 7.8 L/hr for a 65 kg man (115). The shape of the plasma concentration-time curves for MZP suggested that the drug undergoes enterohepatic recycling, as do pyrimethamine, metoprine and etoprine (75). The N-oxides of MZP may be eliminated in the bile and reduced in the gut to the parent drug, which is subsequently absorbed back into the circulation.

Patients 6, 7 and 8 showed MZP plasma concentrations of 0.20, 0.04 and 0.15 mg/L respectively 48 hours after the start of infusion of MZPES at 50 mg/m². The ID_{50} of MZP against rat liver DHFR was 1.3 nM (4×10^{-4} mg/L, ref 87). Thus even after low dosage of MZPES plasma levels of MZP well in excess of that needed to inhibit DHFR were maintained for a long period. Furthermore, MZP is able to reach concentrations in tissues 4 to 14-fold those found in plasma (Section 3.10). No tumour response was observed in any patient entered into the phase I trial however, and further studies on the biochemical pathways involving folate metabolism will be necessary before the lack of sensitivity can be explained.

MZP is probably excreted both as unchanged drug and metabolites in the urine and faeces of man, as in the rat (Section 3.9). Because MZP was metabolized in the liver (Section 3.8) and eliminated *via* the kidneys (Section 3.9) it would be appropriate to evaluate the drug in patients with hepatic and renal dysfunction, although this may pose an ethical problem.

MZP showed only weak activity as an inhibitor of HMT (88) and in the phase I clinical trial no histamine-related effects (neurological or dermal) were observed. Nausea and vomiting were however experienced by some patients receiving the highest dose (460 mg/m²). The AUC above a certain threshold may be more relevant to toxicity than the absolute value of this pharmacokinetic parameter.

The onset, duration and intensity of action of a drug are controlled by the rate at which it reaches its

site of action and by the concentration of the drug at the receptor. It is difficult to assess the distribution of a drug because its fate is determined by a series of dynamic interactions and tissue levels cannot usually be measured directly in man. The physiological disposition of a drug is controlled by the 3 major processes of absorption, distribution into and within tissues, and elimination. Pharmacokinetics is a mathematical consideration of these processes which relates blood concentration and excretion to time. Pharmacokinetic models envisage a drug distributed within a series of interconnected compartments, which are not necessarily actual body compartments, but which do relate to discrete pools of drug availability. This thesis presents a preliminary account of the pharmacokinetics and metabolic transformations of MZPES.

The phase I trial as reported in this thesis did not establish the maximum tolerated dose of MZPES. Further dose escalation should however define the plasma level of the drug which is toxic to normal cells in man. The therapeutic index of most anticancer drugs is low (84) and a phase II trial of MZPES may allow definition of a margin between efficacy and adverse effects. A study of MZPES in patients with different but measurable malignant conditions may clarify relationships between *in vitro* sensitivity, drug scheduling, routes of administration and clinical responses.

APPENDIX 1

MZP excretion balance study in rats - details of recoveries

Rat no. 1 Weight 200 g MZP dose 10 mg (50 mg/kg)

Urine

Time (hr)	Urine vol(ml)	MZP		MAP	
		Assay (mg/L)	% of dose	Assay (mg/L)	% of dose
0-21	9.9	1.46	0.14	7.72	0.76
21-48	10.3	1.26	0.13	6.06	0.62

Faeces

Time (hr)	Faeces wt (g)	MZP		MAP	
		Assay (mg/kg)	% of dose	Assay (mg/kg)	% of dose
0-21	8.7	0.00	0.00	12.53	1.09
21-48	12.7	0.00	0.00	7.17	0.91

Rat no. 2. Weight 245 g MZP dose 12.25 mg (50 mg/kg)

Urine

Time (hr)	Urine vol(ml)	MZP		MAP	
		Assay (mg/L)	% of dose	Assay (mg/L)	% of dose
0-24	12.0	2.99	0.29	20.15	1.93
24-48	12.0	4.78	0.46	29.30	2.81
48-72	20.0	0.09	0.02	2.09	0.33

Faeces

Time (hr)	Faeces wt (g)	MZP		MAP	
		Assay (mg/kg)	% of dose	Assay (mg/kg)	% of dose
0-24	3.9	0.94	0.03	22.60	0.72
24-48	12.1	0.41	0.04	19.74	1.95
48-72	14.6	0.00	0.00	1.80	0.21

Rat no. 1	Weight 200 g	MZP dose 20 mg (100 mg/kg)			
Urine					
Time (hr)	Urine vol(ml)	MZP		MAP	
		Assay (mg/L)	% of dose	Assay (mg/L)	% of dose
0-21	7.2	57.40	2.07	30.20	1.09
21-48	7.0	25.70	0.90	141.50	4.96
Faeces					
Time (hr)	Faeces wt (g)	MZP		MAP	
		Assay (mg/kg)	% of dose	Assay (mg/kg)	% of dose
0-21	2.4	63.30	0.76	101.67	1.22
21-48	0.7	22.86	0.08	231.43	0.81

Rat no. 2 Weight 245 g MZP dose 24.5 mg (100 mg/kg)

Urine

Time (hr)	Urine vol(ml)	MZP		MAP	
		Assay (mg/L)	% of dose	Assay (mg/L)	% of dose
0-24	35.0	0.00	0.00	23.60	3.37
24-48	16.2	0.00	0.00	14.30	0.95
48-72	19.2	0.00	0.00	1.10	0.09

Faeces

Time (hr)	Faeces wt (g)	MZP		MAP	
		Assay (mg/kg)	% of dose	Assay (mg/kg)	% of dose
0-24	9.3	24.50	0.93	272.13	10.33
24-48	13.0	0.00	0.00	11.12	0.59
48-72	10.8	0.00	0.00	0.00	0.00

APPENDIX 2

MZP plasma pharmacokinetics in mice - results from the IP and PO studies

Time (hr)	Plasma MZP conc (mg/L)					Mean	SD
	Mouse						
	1	2	3	4	5		
	MZP dose 20 mg/kg IP						
0.25	6.83	7.86	8.55	8.19	6.91	7.67	0.77
0.50	6.68	4.65	6.98	5.14	5.29	5.75	1.02
0.75	6.69	6.24	7.11	7.57	6.68	6.86	0.50
1.00	5.12	4.97	6.29	6.19	5.26	5.57	0.63
1.50	3.40	5.60	5.85	3.82	4.10	4.55	1.10
2.00	4.09	4.51	5.41	1.54	4.19	3.95	1.44
2.50	4.07	4.39	3.85	3.82	3.21	3.87	0.43
3.00	3.15	3.37	4.04	3.38	3.97	3.58	0.40
4.00	1.60	2.52	3.68	2.73	3.34	2.77	0.80
6.00	1.76	2.02	2.12	2.51	2.53	2.19	0.33
8.00	1.59	1.63	1.37	1.61	2.12	1.66	0.28
12.00	0.71	0.98	0.72	0.47	1.23	0.82	0.29
24.00	0.23	0.27	0.00	0.23	0.00	0.15	0.13
	MZP dose 10 mg/kg IP						
0.25	3.05	2.80	2.05	2.57	2.54	2.60	0.37
0.50	2.64	2.39	2.28	2.13	2.37	2.36	0.19
0.75	2.02	1.81	1.96	2.19	1.99	1.99	0.14
1.00	2.65	2.03	1.78	1.99	1.76	2.04	0.36
1.50	1.37	1.55	1.51	1.61	1.37	1.48	0.11
2.00	1.80	1.44	1.65	1.29	1.54	1.54	0.20
2.50	1.31	1.36	1.04	1.18	1.05	1.19	0.15
3.00	1.46	1.23	1.13	1.18	1.60	1.32	0.20
4.00	1.03	1.00	1.12	0.85	1.09	1.02	0.11
6.00	0.81	0.70	0.67	0.85	0.66	0.74	0.09
8.00	0.45	0.53	0.47	0.37	0.16	0.40	0.14
12.00	0.30	0.23	0.20	0.00	0.00	0.15	0.14
24.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Time (hr)	Plasma MZP conc (mg/L)					Mean	SD
	Mouse						
	1	2	3	4	5		
	MZP dose 10 mg/kg PO						
0.25	1.76	1.43	2.15	1.85	1.63	1.76	0.27
0.50	1.60	1.77	1.91	1.25	1.57	1.62	0.25
0.75	2.03	1.63	2.06	1.82	1.92	1.89	0.18
1.00	1.63	2.73	1.91	1.96	2.13	2.07	0.41
1.50	1.49	1.09	1.81	0.99	1.51	1.38	0.34
2.00	1.72	1.89	1.82	1.31	1.26	1.60	0.29
2.50	1.10	0.94	0.88	0.99	0.90	0.96	0.09
3.00	1.54	1.31	1.02	1.37	1.57	1.36	0.22
4.00	1.36	1.55	0.79	1.32	1.68	1.14	0.38
6.00	1.16	1.15	1.13	1.18	0.72	1.07	0.20
8.00	0.68	1.16	0.36	0.61	0.72	0.71	0.29
12.00	0.32	0.47	0.04	0.27	0.41	0.30	0.17
24.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

APPENDIX 3

MZP and MAP tissue pharmacokinetics in mice
- results from the IP study at 20 mg/kg dosage

Time (hr)	Mouse 1	Mouse 2	Mean
	Liver MZP conc (mg/kg)		
0.5	38.85	38.00	38.43
1.0	28.30	28.70	28.50
1.5	27.50	28.40	27.95
2.0	17.60	20.10	18.85
4.0	11.55	10.25	10.90
6.0	4.80	5.80	5.30
8.0	2.75	7.20	4.98
12.0	0.00	2.45	1.23
24.00	0.00	0.75	0.38
48.00	0.00	0.00	0.00
	Liver MAP conc (mg/kg)		
0.5	19.90	24.05	21.98
1.0	28.90	28.75	28.83
1.5	21.95	25.35	23.65
2.0	21.65	19.00	20.33
4.0	14.70	19.70	17.20
6.0	16.85	20.10	18.48
8.0	19.50	19.30	19.40
12.0	14.85	12.80	13.83
24.0	4.05	26.00	4.05
48.0	0.00	0.00	0.00
	Brain MZP conc (mg/kg)		
0.5	38.75	33.45	36.10
1.0	33.75	32.80	33.28
1.5	30.90	25.35	28.13
2.0	23.70	26.40	25.05
4.0	15.75	18.15	16.95
6.0	9.90	13.15	11.53
8.0	9.70	7.70	8.70
12.0	4.75	4.35	4.55
24.0	0.95	3.95	2.45
48.0	0.65	0.95	0.80

Time (hr)	Mouse 1	2	Mean
	Brain MAP conc (mg/kg)		
0.5	2.80	1.85	2.33
1.0	1.70	2.25	1.98
1.5	2.05	1.45	1.75
2.0	2.15	2.55	2.35
4.0	3.65	3.40	3.53
6.0	4.60	4.10	4.35
8.0	5.05	4.90	4.98
12.0	4.85	4.80	4.83
24.0	1.70	7.40	1.70
48.0	0.00	0.00	0.00
	Lung MZP conc (mg/kg)		
0.5	46.25	66.45	56.35
1.0	59.75	53.55	56.65
1.5	57.60	71.35	64.48
2.0	46.85	43.40	45.13
4.0	34.95	43.00	38.98
6.0	20.50	28.25	24.38
8.0	26.05	17.70	21.88
12.0	8.60	9.85	9.23
24.0	2.30	6.05	4.18
48.0	2.00	0.30	1.15
	Lung MAP conc (mg/kg)		
0.5	6.05	3.85	4.95
1.0	3.75	4.25	4.00
1.5	4.80	5.45	5.13
2.0	7.90	5.20	6.55
4.0	13.55	14.05	13.80
6.0	13.40	16.70	15.05
8.0	21.60	16.20	18.90
12.0	13.95	14.35	14.15
24.0	3.10	20.65	3.10
48.0	0.55	0.35	0.45
	Heart MZP conc (mg/kg)		
0.5	22.90	20.90	21.90
1.0	13.80	16.75	15.28
1.5	11.80	12.90	12.35
2.0	8.10	9.50	8.80
4.0	6.10	5.55	5.83
6.0	3.75	6.90	5.33
8.0	3.20	2.10	2.65
12.0	0.75	2.35	1.55
24.0	0.60	1.45	1.03
48.0	0.40	0.00	0.20

Time (hr)	Mouse 1	2	Mean
	Heart MAP conc (mg/kg)		
0.5	8.85	9.40	9.13
1.0	8.75	7.40	8.08
1.5	10.15	8.40	9.28
2.0	7.30	7.50	7.40
4.0	6.35	5.15	5.75
6.0	7.20	8.30	7.75
8.0	8.75	5.70	7.23
12.0	5.25	5.20	5.23
24.0	1.90	11.50	1.90
48.0	1.25	0.00	0.63
	Kidney MZP conc (mg/kg)		
0.5	39.70	37.70	38.70
1.0	27.60	35.10	31.35
1.5	37.35	32.10	34.73
2.0	22.85	21.15	22.00
4.0	9.20	10.70	9.95
6.0	6.15	10.20	8.18
8.0	7.65	7.45	7.55
12.0	3.15	3.20	3.18
24.0	1.95	3.85	2.90
48.0	3.25	0.00	1.63
	Kidney MAP conc (mg/kg)		
0.5	14.95	13.65	14.30
1.0	16.35	17.65	17.00
1.5	21.65	21.45	21.55
2.0	14.75	18.05	16.40
4.0	19.90	19.10	19.50
6.0	15.60	23.20	19.40
8.0	21.00	17.20	19.10
12.0	14.70	13.40	14.05
24.0	5.70	25.55	5.70
48.0	1.30	0.00	0.65

APPENDIX 4

Plasma levels of MZP determined in patients in the Birmingham arm of the phase I trial

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 1 MZPES dose (mg/m²) 27			
0.88	0.504	- 0.685	0.319	- 1.141
1.28	0.337	- 1.088	0.317	- 1.150
1.65	0.302	- 1.197	0.314	- 1.158
2.02	0.258	- 1.355	0.311	- 1.166
2.52	0.253	- 1.376	0.308	- 1.177
3.00	0.282	- 1.266	0.305	- 1.188
4.05	0.249	- 1.392	0.298	- 1.211
6.15	0.288	- 1.247	0.285	- 1.257
8.00	0.311	- 1.168	0.273	- 1.297
12.18	0.257	- 1.361	0.249	- 1.388
	Patient no. 2 MZPES dose (mg/m²) 27			
1.35	0.159	- 1.842	0.157	- 1.853
1.67	0.164	- 1.811	0.154	- 1.868
2.00	0.142	- 1.952	0.152	- 1.883
2.48	0.144	- 1.938	0.149	- 1.905
2.98	0.129	- 2.052	0.145	- 1.929
3.98	0.172	- 1.763	0.139	- 1.975
5.98	0.122	- 2.104	0.126	- 2.068
7.98	0.113	- 2.180	0.115	- 2.161
	Patient no. 3 MZPES dose (mg/m²) 27			
1.48	0.284	- 1.259	0.266	- 1.325
1.83	0.297	- 1.214	0.264	- 1.331
2.18	0.267	- 1.321	0.262	- 1.338
2.53	0.243	- 1.417	0.261	- 1.334
3.03	0.240	- 1.427	0.258	- 1.353
4.08	0.233	- 1.459	0.253	- 1.373
8.00	0.235	- 1.448	0.236	- 1.445
12.18	0.226	- 1.487	0.218	- 1.523

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 3 MZPES dose mg/m²) 27			
1.05	0.571	- 0.560	0.614	- 0.487
1.42	0.313	- 1.162	0.294	- 1.226
1.68	0.248	- 1.396	0.250	- 1.387
2.05	0.239	- 1.433	0.234	- 1.453
2.55	0.220	- 1.514	0.227	- 1.482
6.05	0.196	- 1.630	0.197	- 1.627
7.97	0.183	- 1.701	0.182	- 1.706
	Patient no. 8 MZPES dose (mg/m²) 50			
0.95	1.061	0.059	0.852	- 0.160
3.00	0.364	- 1.011	0.399	- 0.919
4.02	0.376	- 0.979	0.357	- 1.029
6.03	0.293	- 1.229	0.330	- 1.109
8.00	0.287	- 1.250	0.317	- 1.148
24.42	0.288	- 1.245	0.239	- 1.430
48.25	0.148	- 1.911	0.159	- 1.839
	Patient no. 9 MZPES dose (mg/m²) 67			
0.87	3.956	1.375	2.427	0.887
1.27	0.726	- 0.320	0.948	- 0.054
1.67	0.505	- 0.684	0.566	- 0.568
2.02	0.509	- 0.675	0.473	- 0.749
2.52	0.472	- 0.752	0.436	- 0.829
2.98	0.405	- 0.905	0.427	- 0.851
3.97	0.413	- 0.886	0.417	- 0.875
6.03	0.424	- 0.859	0.400	- 0.916
7.95	0.383	- 0.960	0.385	- 0.954
11.95	0.324	- 1.127	0.356	- 1.034
24.08	0.299	- 1.207	0.279	- 1.277
48.17	0.170	- 1.775	0.172	- 1.758
	Patient no.11 MZPES dose (mg/m²) 67			
1.02	1.700	0.530	1.654	0.503
1.33	0.792	- 0.234	1.472	0.387
1.68	0.866	- 0.144	1.323	0.280
2.12	0.963	- 0.038	1.194	0.177
2.55	1.078	0.075	1.108	0.103
3.07	1.056	0.054	1.038	0.038
4.00	0.899	- 0.107	0.963	- 0.038
6.00	0.904	- 0.101	0.877	- 0.132
8.03	0.799	- 0.224	0.815	- 0.205
12.05	0.777	- 0.252	0.709	- 0.344
23.92	0.430	- 0.844	0.471	- 0.753
48.00	0.211	- 1.558	0.205	- 1.583

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 12 MZPES dose (mg/m²) 67			
1.00	1.925	0.655	1.372	0.316
1.38	1.019	0.019	1.190	0.174
1.75	0.938	- 0.064	1.059	0.058
2.05	0.967	- 0.034	0.979	- 0.022
2.53	0.918	- 0.086	0.884	- 0.124
2.97	0.842	- 0.173	0.823	- 0.195
4.13	0.672	- 0.397	0.731	- 0.314
6.00	0.678	- 0.389	0.670	- 0.400
7.70	0.654	- 0.425	0.642	- 0.443
11.70	0.574	- 0.555	0.593	- 0.523
23.57	0.475	- 0.745	0.473	- 0.749
48.00	0.298	- 1.212	0.297	- 1.214
	Patient no. 12 MZPES dose (mg/m²) 83			
1.25	1.235	0.211	1.154	0.143
1.72	1.134	0.125	1.098	0.094
2.12	0.939	- 0.063	1.056	0.055
2.50	1.019	0.019	1.020	0.020
2.85	1.021	0.021	0.989	- 0.011
3.23	0.983	- 0.017	0.959	- 0.042
4.10	0.865	- 0.145	0.899	- 0.106
6.13	0.743	- 0.297	0.796	- 0.228
8.05	0.594	- 0.522	0.730	- 0.315
12.05	0.782	- 0.246	0.639	- 0.448
21.47	0.336	- 1.092	0.508	- 0.678
47.80	0.323	- 1.130	0.283	- 1.264
	Patient no. 13 MZPES dose (mg/m²) 83			
0.93	1.742	0.555	1.477	0.390
1.47	1.008	0.007	1.115	0.109
1.83	1.004	0.004	1.003	0.003
2.23	0.952	- 0.049	0.934	- 0.068
2.55	0.932	- 0.070	0.902	- 0.104
3.05	0.860	- 0.151	0.871	- 0.138
4.03	0.847	- 0.166	0.839	- 0.176
6.02	0.814	- 0.206	0.795	- 0.229
8.05	0.747	- 0.292	0.756	- 0.280
12.10	0.655	- 0.424	0.683	- 0.381
23.80	0.521	- 0.653	0.509	- 0.674
48.05	0.277	- 1.284	0.277	- 1.282

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 13	MZPES dose (mg/m²)	83	
0.77	2.908	1.067	2.541	0.933
1.02	1.163	0.151	1.251	0.224
1.33	1.005	0.005	0.999	- 0.001
1.67	1.008	0.007	0.962	- 0.039
2.02	0.989	- 0.011	0.952	- 0.049
2.50	1.107	0.102	0.941	- 0.060
3.00	1.121	0.114	0.931	- 0.072
4.10	0.867	- 0.143	0.908	- 0.096
6.05	0.897	- 0.109	0.869	- 0.140
7.97	0.904	- 0.101	0.833	- 0.183
11.00	0.725	- 0.322	0.778	- 0.251
24.15	0.583	- 0.540	0.580	- 0.546
48.23	0.338	- 1.085	0.338	- 1.085
	Patient no. 14	MZPES dose (mg/m²)	83	
1.20	4.794	1.567	3.613	1.285
1.45	1.103	0.098	1.371	0.315
1.68	1.016	0.016	0.964	- 0.036
2.03	0.734	- 0.309	0.860	- 0.151
2.52	0.915	- 0.089	0.841	- 0.173
3.02	0.907	- 0.098	0.832	- 0.184
3.98	0.848	- 0.165	0.815	- 0.205
6.00	0.738	- 0.304	0.780	- 0.248
8.00	0.717	- 0.333	0.747	- 0.291
11.95	0.698	- 0.360	0.686	- 0.376
24.00	0.484	- 0.727	0.530	- 0.635
48.08	0.331	- 1.106	0.316	- 1.153
	Patient no. 14	MZPES dose (mg/m²)	83	
1.40	1.808	0.592	1.543	0.434
1.78	1.129	0.121	1.216	0.195
2.15	0.905	- 0.100	1.019	0.018
2.52	0.981	- 0.019	0.895	- 0.111
3.03	0.751	- 0.286	0.794	- 0.231
4.02	0.672	- 0.398	0.707	- 0.347
6.00	0.629	- 0.464	0.650	- 0.430
8.00	0.616	- 0.485	0.618	- 0.482
12.10	0.565	- 0.572	0.559	- 0.582
23.67	0.441	- 0.820	0.422	- 0.863
47.92	0.230	- 1.472	0.234	- 1.452

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 15		MZPES dose (mg/m²)	83
1.12	1.729	0.547	1.332	0.286
1.50	0.871	- 0.138	0.991	- 0.009
1.95	0.767	- 0.265	0.790	- 0.236
2.25	0.692	- 0.369	0.719	- 0.330
2.63	0.695	- 0.365	0.667	- 0.405
3.03	0.638	- 0.449	0.637	- 0.452
3.90	0.637	- 0.452	0.607	- 0.500
5.92	0.628	- 0.466	0.579	- 0.546
7.85	0.597	- 0.517	0.559	- 0.581
11.88	0.491	- 0.712	0.519	- 0.655
21.00	0.321	- 1.136	0.440	- 0.822
47.25	0.309	- 1.176	0.272	- 1.302
	Patient no. 17		MZPES dose (mg/m²)	105
1.15	1.676	0.516	1.676	0.516
1.50	1.052	0.050	1.372	0.316
1.75	0.948	- 0.054	1.257	0.228
2.10	1.048	0.047	1.168	0.155
2.53	0.969	- 0.032	1.116	0.109
3.03	1.087	0.083	1.087	0.083
4.05	1.088	0.084	1.057	0.056
6.03	1.163	0.151	1.017	0.017
8.03	0.994	- 0.007	0.979	- 0.021
12.05	0.930	- 0.073	0.908	- 0.097
27.03	0.639	- 0.449	0.683	- 0.381
48.87	0.465	- 0.766	0.452	- 0.795
	Patient no. 18		MZPES dose (mg/m²)	105
1.20	1.974	0.680	1.974	0.680
1.43	1.267	0.237	1.267	0.237
1.72	0.329	0.284	1.201	0.183
2.07	1.295	0.258	1.193	0.177
2.50	0.990	- 0.010	1.187	0.171
3.03	1.257	0.229	1.179	0.165
4.02	1.236	0.212	1.165	0.153
6.00	0.966	- 0.035	1.137	0.129
8.00	1.220	0.199	1.110	0.104
12.00	0.960	- 0.041	1.057	0.055
26.83	0.928	- 0.075	0.882	- 0.126
48.65	0.670	- 0.401	0.675	- 0.393

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 19		MZPES dose (mg/m²)	105
0.88	1.719	0.542	1.732	0.549
1.35	1.250	0.223	1.193	0.176
1.67	0.971	- 0.030	1.014	0.014
2.00	0.921	- 0.082	0.909	- 0.096
2.50	0.798	- 0.226	0.828	- 0.188
3.00	0.901	- 0.105	0.791	- 0.234
4.00	0.758	- 0.277	0.760	- 0.275
6.25	0.731	- 0.314	0.724	- 0.323
8.00	0.626	- 0.469	0.699	- 0.358
26.55	0.428	- 0.849	0.486	- 0.722
52.05	0.317	- 1.150	0.295	- 1.222
	Patient no. 21		MZPES dose (mg/m²)	125
1.08	1.858	0.619	1.858	0.619
1.38	1.011	0.011	1.011	0.011
1.68	0.975	- 0.025	0.906	- 0.098
2.02	0.935	- 0.067	0.891	- 0.116
2.52	0.818	- 0.201	0.885	- 0.122
3.00	0.891	- 0.115	0.881	- 0.126
4.00	0.827	- 0.191	0.873	- 0.135
6.00	0.857	- 0.155	0.858	- 0.154
8.02	0.959	- 0.042	0.842	- 0.172
12.25	0.705	- 0.350	0.810	- 0.211
24.03	0.825	- 0.193	0.727	- 0.319
48.53	0.557	- 0.585	0.581	- 0.543
	Patient no. 22		MZPES dose (mg/m²)	150
1.43	9.190	2.218	7.606	2.029
2.02	2.553	0.937	2.978	1.091
2.52	2.259	0.815	2.177	0.778
3.03	2.148	0.764	1.970	0.678
3.98	1.795	0.585	1.876	0.629
6.00	1.736	0.552	1.785	0.580
8.00	1.822	0.600	1.703	0.532
12.07	1.581	0.458	1.547	0.436
24.58	1.130	0.122	1.151	0.141

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 23		MZPES dose (mg/m²)	150
0.72	3.248	1.178	2.638	0.970
1.00	1.976	0.681	2.177	0.778
1.33	1.805	0.591	1.890	0.636
1.67	1.799	0.587	1.739	0.553
2.00	1.678	0.518	1.661	0.507
2.50	1.528	0.424	1.598	0.469
3.00	1.544	0.434	1.562	0.446
4.02	1.728	0.547	1.509	0.411
6.02	1.432	0.359	1.420	0.351
8.02	1.506	0.409	1.337	0.291
10.90	1.012	0.012	1.226	0.204
25.78	0.715	- 0.336	0.783	- 0.244
49.12	0.413	- 0.886	0.388	- 0.947
	Patient no. 28		MZPES dose (mg/m²)	210
1.08	9.381	2.239	7.960	2.074
1.35	3.738	1.319	4.223	1.441
1.67	3.211	1.166	3.147	1.147
2.00	3.255	1.180	2.904	1.066
2.50	3.012	1.103	2.816	1.035
3.03	2.896	1.063	2.771	1.019
4.00	3.092	1.129	2.697	0.992
6.02	2.746	1.010	2.550	0.936
8.00	2.376	0.865	2.414	0.881
11.87	1.831	0.605	2.168	0.774
24.02	1.402	0.338	1.548	0.437
47.85	0.860	- 0.151	0.799	- 0.224
	Patient no. 28		MZPES dose (mg/m²)	210
0.82	5.310	1.670	5.304	1.669
1.22	3.564	1.271	3.679	1.303
1.65	3.090	1.128	2.977	1.091
2.00	2.709	0.996	2.732	1.005
2.53	2.580	0.948	2.571	0.944
2.98	2.711	0.997	2.510	0.920
4.00	2.311	0.838	2.435	0.890
6.00	2.543	0.933	2.327	0.845
21.45	0.556	0.442	1.646	0.499
45.45	0.981	- 0.019	0.962	- 0.039

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 29		MZPES dose (mg/m²)	210
1.02	4.549	1.515	4.474	1.498
1.43	2.322	0.842	2.339	0.850
1.67	2.168	0.774	2.165	0.772
2.00	2.214	0.795	2.101	0.742
2.52	2.392	0.872	2.065	0.725
3.00	2.801	1.030	2.039	0.712
4.02	2.821	1.037	1.985	0.686
6.02	1.866	0.624	1.883	0.633
8.02	2.414	0.881	1.786	0.580
11.85	1.641	0.495	1.615	0.479
23.17	1.188	0.172	1.198	0.181
49.00	0.607	- 0.499	0.606	- 0.500
	Patient no. 30		MZPES dose (mg/m²)	210
0.98	30.567	3.420	24.234	3.188
1.38	3.275	1.186	4.244	1.446
1.68	2.580	0.948	2.426	0.886
2.02	2.547	0.935	2.076	0.730
2.53	2.279	0.824	1.996	0.691
3.00	2.094	0.739	1.966	0.676
4.00	1.810	0.593	1.911	0.648
6.02	1.768	0.570	1.804	0.590
8.00	1.467	0.383	1.704	0.533
23.85	1.098	0.093	1.083	0.080
47.85	0.554	- 0.591	0.546	- 0.606
	Patient no. 30		MZPES dose (mg/m²)	250
1.03	19.802	2.986	15.375	2.733
1.35	3.932	1.369	4.597	1.525
1.67	3.358	1.211	3.296	1.193
2.00	3.057	1.117	3.113	1.136
2.50	2.974	1.090	3.048	1.114
3.00	3.082	1.126	3.000	1.099
4.00	2.948	1.081	2.909	1.068
6.00	2.627	0.966	2.734	1.006
8.00	2.471	0.905	2.571	0.944
24.23	1.776	0.574	1.557	0.442
48.07	0.705	- 0.350	0.745	- 0.294

Time (hr)	Measured mean conc (mg/L)	Measured Ln conc	Fitted conc (mg/L)	Fitted Ln conc
	Patient no. 32		MZPES dose (mg/m²)	250
1.00	5.386	1.684	4.741	1.556
1.35	3.241	1.176	4.233	1.443
1.68	3.316	1.199	3.919	1.366
2.02	3.456	1.240	3.703	1.309
2.52	3.734	1.317	3.505	1.254
3.02	3.803	1.336	3.390	1.221
4.08	3.889	1.358	3.260	1.182
6.08	3.402	1.224	3.131	1.141
7.67	3.327	1.202	3.047	1.114
11.12	2.655	0.976	2.876	1.056
23.08	2.665	0.976	2.354	0.856
47.08	1.514	0.415	1.576	0.455
	Patient no. 36		MZPES dose (mg/m²)	360
0.98	5.790	1.756	5.412	1.689
1.32	4.066	1.403	4.284	1.455
1.67	3.704	1.309	3.711	1.311
2.00	3.458	1.241	3.435	1.234
2.50	3.110	1.135	3.232	1.173
3.03	3.182	1.158	3.128	1.140
4.00	2.815	1.035	3.018	1.105
6.00	3.097	1.130	2.844	1.045
8.00	2.625	0.965	2.683	0.987
24.07	1.702	0.532	1.679	0.518
47.82	0.833	- 0.183	0.840	- 0.175
	Patient no. 37		MZPES dose (mg/m²)	360
0.85	8.976	2.195	8.405	2.129
1.33	6.186	1.822	6.544	1.879
1.67	5.573	1.718	5.736	1.747
2.00	5.372	1.681	5.205	1.650
2.50	5.022	1.614	4.701	1.548
3.00	5.450	1.696	4.410	1.484
4.00	5.255	1.659	4.129	1.418
6.00	3.964	1.377	3.922	1.367
8.00	3.600	1.281	3.798	1.334
10.48	3.597	1.280	3.657	1.297
23.98	3.268	1.184	2.979	1.092
47.98	1.999	0.692	2.070	0.728

APPENDIX 5

Pharmacokinetic parameters evaluated for all patients in the clinical trial (both Birmingham and Charing Cross)

Patient	Dose (mg/m^2)	Cp_{max} (mg/L)	AUC_{trap} (mg L/hr)	Vol central compartment (L)	Apparent Vd (L)	Distribution $t_{\frac{1}{2}}$ (hr)	Elimination $t_{\frac{1}{2}}$ (hr)	Plasma clearance (L/hr)
1	27	0.504	3.449	-	102.324	-	31.710	13.337
2	27	0.269	1.120	-	274.242	-	14.910	40.179
3	27	0.572	3.008	-	166.747	-	37.420	14.960
3	27	0.571	2.790	15.638	135.347	0.171	71.404	15.771
4	27	0.355	1.542	-	83.904	-	10.390	24.643
5	38	1.964	8.444	-	133.551	-	41.020	8.527
6	50	0.672	15.108	-	104.684	-	34.250	4.832
7	50	14.603	11.650	-	332.252	-	22.620	6.867
7	67	1.819	13.593	17.040	190.543	0.218	41.001	7.872
8	50	1.061	13.829	9.972	159.877	0.119	72.618	5.279
9	67	3.956	16.403	3.211	160.846	0.108	30.424	7.803
10	67	0.956	13.919	-	86.900	-	20.902	7.256
11	67	1.700	25.420	37.139	70.623	0.663	20.110	4.170
12	67	1.925	24.387	17.369	109.279	0.164	24.358	5.618
12	83	1.235	23.255	90.365	153.232	1.905	31.760	7.310

Patient	Dose (mg/m^2)	Cp_{max} (mg/L)	AUC_{trap} (mgL/hr)	Vol central Compartment (L)	Apparent Vd (L)	Distribution $t_{\frac{1}{2}}$ (hr)	Elimination $t_{\frac{1}{2}}$ (hr)	Plasma clearance (L/hr)
13	83	1.742	26.507	32.665	126.214	0.186	25.025	6.451
13	83	2.908	30.400	5.304	110.641	0.064	26.820	5.592
14	83	4.794	28.634	1.700	107.119	0.062	28.804	5.378
14	83	1.806	22.583	36.625	148.528	0.505	31.936	6.731
15	83	1.729	19.744	28.983	148.585	0.299	32.594	7.091
16	83	2.634	23.770	16.645	90.124	0.075	30.506	6.815
17	105	1.676	36.145	23.340	107.325	0.146	41.385	4.510
18	105	1.974	44.858	7.610	103.773	0.065	54.942	3.990
19	105	1.719	25.930	47.990	156.258	0.403	32.702	6.942
20	105	2.955	28.113	6.596	127.944	0.154	24.352	7.328
21	125	1.858	36.731	19.642	199.924	0.101	76.670	6.699
22	150	9.190	45.234	3.844	87.635	0.154	24.609	6.915
23	150	3.248	42.668	36.437	97.928	0.274	21.657	5.531
24	177	4.099	45.827	8.905	55.031	0.049	13.300	5.630
25	200	2.540	41.864	75.010	105.799	0.140	34.909	7.930
26	200	4.101	90.840	57.488	77.018	0.585	33.955	3.611
27	200	2.795	43.353	74.524	107.089	0.179	23.710	8.719
28	210	9.380	80.738	3.166	69.135	0.078	19.035	4.236
28	210	5.300	78.508	26.699	88.704	0.275	28.780	4.356

Patient	Dose (mg/m^2)	Cp_{max} (mg/L)	AUC_{trap} (mg L hr)	Vol central Compartment (L)	Apparent Vd (L)	Distribution $t_{\frac{1}{2}}$ (hr)	Elimination $t_{\frac{1}{2}}$ (hr)	Plasma clearance (L/hr)
29	210	4.549	66.226	6.670	95.012	0.041	22.957	5.194
30	210	30.567	74.662	0.808	72.316	0.084	18.269	5.331
30	250	19.802	96.961	1.611	88.155	0.073	23.322	4.889
31	215	2.400	59.000	44.557	109.563	0.063	32.770	5.509
32	250	5.386	118.919	34.670	69.684	0.221	38.691	3.036
33	300	5.357	135.750	63.468	120.760	2.785	131.856	3.735
34	300	8.458	88.119	22.349	70.243	0.290	49.502	4.880
35	360	7.035	134.624	5.400	82.803	0.050	21.126	4.732
36	360	5.790	89.765	45.821	127.061	0.292	24.325	6.662
37	360	8.976	157.062	28.030	74.686	0.329	31.836	3.381
37	400	10.221	126.960	31.647	61.181	0.399	26.269	4.624
38	460	8.888	164.843	48.794	93.465	0.267	22.124	5.217

— calculated as single compartment model

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